Metabolic Pathways

(Second Edition of Chemical *Pathways of Metabolism*)

EDITED BY

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PREFACE

Knowledge of the metabolic processes of living organisms continues to accumulate at a gratifying pace. A wealth of new information has been acquired since the publication of the first edition of the work. Many missing details in the knowledge of particular intermediates and enzymes in established cycles have now been determined. New, hitherto unsuspected metabolic patterns have been discovered for many natural compounds. In many instances these represent alternate pathways to previously established metabolic cycles.

The last few years have seen the discovery of specific coenzyme functions for biotin and for vitamin B_{12}. Important progress has been made in the elucidation of the mechanisms of biosynthesis of the biological macromolecules primarily concerned with the maintenance of life processes, the proteins, and the nucleic acids.

These advances are discussed in this work. In addition separate chapters have been included on the metabolism of the carotenoids, vitamins, and coenzymes.

The purpose of this work remains, as in the first edition, to survey the existing knowledge of the chemical steps in the metabolism of the constituents of major importance in living organisms. In the selection of authors individuals have been chosen who are actively working in each of the areas covered and know the subject matter from personal day-to-day contact with it in the laboratory.

If one is led to wonder as to the reason for the change in title of these volumes, the answer is that the new title, "Metabolic Pathways" is strongly appealing for its brevity and connotations of content.

University of California
School of Medicine
San Francisco, California
June, 1960

DAVID M. GREENBERG

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by W Z HASSID

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LIST OF COMMON ABBREVIATIONS AND SYMBOLS

AMP, GMP, IMP, UMP, CMP	The 5′-phosphates of ribosyl adenine, guanine, hypoxanthine, uracil, and cytosine
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
NMN	Nicotinamide mononucleotide
DPN_{ox}, DPN^+, DPN	Diphosphopyridine nucleotide (cozymase, coenzyme I)
DPN_{red}, DPNH	Reduced form of above
TPN_{ox}, TPN^+, TPN_{red}, TPNH	Triphosphopyridine nucleotide (coenzyme II)
FAD, $FADH_2$	Flavin adenine dinucleotide and its reduced form
CoA, CoASH	Coenzyme A
RSH	Sulfhydryl compounds
GSH, GSSG	Glutathione and its oxidized form
L(SH)(SH), L(S–S)	Lipoic acid, thioctic acid
Kcal	Kilocalories
Kj	Kilojoules
Q_{O_2}, $Q_{acetate}$, etc	Metabolic quotients expressed in µl metabolite/mg dry weight/hr
ΔF	Increment of free energy
ΔF^0	Standard free energy change
$\Delta F'$	Standard free energy change at pH 7
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
P	Inorganic phosphate
PP	Inorganic pyrophosphate
PPP	Inorganic triphosphate
PLP	Pyridoxal phosphate
EDTA	Ethylenediamine tetraacetic acid

TENTATIVE CONTENTS OF VOLUME 2

Free Energy and Entropy in Metabolism

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Page

I. Introduction

The most useful concept that biochemists have acquired from thermodynamics is that of free energy By considering the free energy change of a reaction one can tell whether it may proceed spontaneously or whether it must be "driven" by other reactions. Further, one can calculate the amount of energy given off by a reaction or required by it; and this is a most important feature of many reactions. From free energy data one can easily calculate equilibrium constants and electromotive forces

In this chapter, an attempt will first be made to introduce the concept of free energy in a simple, descriptive way. The various methods of obtaining free energy data will be shown. Also, this section will provide relations between free energy and other quantities of direct interest, such as equilibrium constants, heats of reactions, and electromotive forces. The remainder of the chapter will be devoted to applications of thermodynamics which will serve to indicate areas of biochemistry in which such information is useful The flow of energy through photosynthesis, assimilation into chemically stored forms such as starch, utilization of components with production of energy-rich phosphate compounds, and, finally, use for synthesis or work of various sorts will be mentioned It is hoped that this presentation will provide a basis for understanding thermodynamic treatments in other chapters of this book and elsewhere in biochemistry

Entropy changes are also of interest to biochemists, since such changes may give an insight into structural changes of the products relative to the reactants A final section of this chapter will deal with applications of entropy in biochemistry

Three types of information will be treated very briefly. First, little attempt will be made to relate free energy to other thermodynamic quantities Many excellent books and articles on thermodynamics, some especially for biochemists, have appeared and may be recommended to those who are not familiar with the fundamental relations (*1–4*) Second, certain subjects such as applications of free energy in carbohydrate metabolism and protein synthesis are discussed in other chapters of this book, and therefore they will not be discussed at length in this chapter. Third, the eventual utilization of energy for work is outside the scope of this treatise.

II. Free Energy and Its Determination

A. The Nature of Free Energy

When we consider the energy changes involved in a reaction such as the oxidation of glucose or in a process like the absorption of light in

photosynthesis, we note that chemical energy is given up as heat or that the energy of light is transformed into chemical energy. Such qualitative statements are not nearly as useful as quantitative information Energy changes should be expressed quantitatively if they are to provide the maximum information regarding a reaction The most useful way of expressing them is in terms of ΔF, the *free energy change* of the reaction. One may think of free energy changes in the following way· every compound may be considered to have a definite amount of free energy (F) stored in it under any set of conditions When certain compounds (reactants) are converted into other compounds (products), the difference (Δ) in the free energies of products and reactants is released if the products have less free energy than the reactants, or energy must be put in if the opposite is the case This difference, ΔF, is the free energy change of the reaction, per mole

$$\Delta F = \text{free energy of products} - \text{free energy of reactants}$$

The point of greatest importance is that for the reaction to proceed spontaneously as written, without putting in outside energy (and at constant temperature and pressure), ΔF must be negative; that is, the products must possess less free energy than the reactants One may say that reactions only go "down hill" energetically of their own accord—from compounds of higher to those of lower free energy. Processes that have a positive ΔF must be supplied with free energy greater than ΔF from another source if they are to proceed They do not occur spontaneously For example, formation of pyrophosphate from phosphate has a ΔF greater than 0 (+9,000 cal per mole) One could treat a solution of phosphate with catalysts like purified enzymes and the formation of pyrophosphate would not occur to an appreciable extent However the reaction in the reverse direction can occur, since ΔF of hydrolysis of pyrophosphate is −9,000 cal In the intermediate case, when ΔF of a reaction is 0, the reaction tends to go equally in both directions The reactants and products are at equilibrium

It might be helpful in discussing the nature of ΔF to mention its close relation to the equilibrium constant and the mass action law In a reaction at equilibrium



$$A + B \rightleftharpoons C + D \qquad (1)$$

$(C)(D)/(A)(B)$ equals K, where (C), (D), (A), and (B) are concentrations and K is the equilibrium constant. When this ratio of products to reactants equals K the reaction is at equilibrium, when it is greater than K the reaction tends to go to the left, and when the ratio is less than K the reaction tends to go to the right; these correspond respectively to conditions of ΔF equal to 0, ΔF greater than 0, and ΔF less than 0

It can be seen that the ΔF of a reaction must depend not only on the chemical structures of reactants and products but also on their concentrations, because the direction in which the reaction proceeds depends on these concentrations. It is not necessary to record ΔF for all possible concentrations since if ΔF is known for one set of conditions it can be calculated for others as described below. Therefore, ΔF is recorded when reactants and products are in certain standard conditions which are: liquids or solids, pure gases at 1 atmosphere, and substances in solution at 1 M concentration, at a definite temperature, usually 25°. Under these conditions concentrations are defined as equal to unity. This ΔF is written ΔF^0 and is called the standard free energy change. It is important not to use ΔF^0 in place of the ΔF calculated for the actual experimental conditions because ΔF^0 has no direct application and is the wrong val For example, ΔF^0 of oxidation of ½ N_2 to NO_3^- by O_2 equals +1780 c but ΔF under actual conditions in the bacterial cell is −7870 cal (6 An organism that operated under the standard conditions at which ΔF is defined would fix little N_2. The method of calculating ΔF from ΔF will be presented later in this chapter.

The second feature of interest is that ΔF is equal to the maximum energy "free" to do work obtainable from a reaction at constant temperature and pressure. For example, if a perfectly efficient man oxidized 1 mole of solid glucose with O_2 to CO_2 (gases at 1 atmosphere) and liquid water, he could do a maximum of 688,000 cal of work because ΔF^0 of the reaction equals −688,000 cal. ΔF does not include work done by any necessary expansion or contraction against external pressure during th reaction—in this case the volume change from O_2 and glucose to equiva lent amounts of CO_2 and H_2O.

ΔF depends only on the products and reactants and not on the reaction pathway. For example, ΔF of the oxidation of glucose is the same whether the sugar is burned in a flame or metabolized in the body so long as the same initial and final conditions are obtained. No useful work is done in the first case and only heat is released, but ΔF of the reaction is the same, because it is equal to the maximum possible work obtainable and not to the actual work obtained.

The maximum heat that is released by a reaction at constant pressure is $-\Delta H$, and this is not necessarily the same as the maximum possible work, $-\Delta F$. As examples, a number of compounds including trichloroacetic acid and ammonium sulfate dissolve spontaneously in water so ΔF is negative; but the solution becomes cooler, and therefore at constant temperature heat would be absorbed, and ΔH must be positive. ΔF and ΔH may differ by plus or minus several thousand calories, as in the case of glycolysis, where ΔF is −35,000 cal and ΔH is −24,000 cal (6), one-

third less The difference between ΔH and ΔF is equal to $T\Delta S$ where ΔS is the difference in entropy of products and reactants.

$$\Delta H = \Delta F + T\Delta S \quad (2)$$

Entropy will be discussed in the final section of this chapter.

The free energy of a reaction is useful for determination of the direction of a reaction and the maximum energy yield, and in several other ways:

1 It can be used to calculate the equilibrium constant.

2 It can be used to calculate the electromotive force of an oxidation-reduction reaction

3. It is useful in calculating other thermodynamic quantities

4. By combining ΔF values of several reactions ΔF values of new reactions may be determined.

5 It provides a criterion for considering the possibility of occurrence of biochemical pathways

Thermodynamics and a knowledge of the free energy of reactions is certainly useful and helpful but gives a far from complete picture of a biological situation. Although living systems must obey the laws of thermodynamics, these laws do not provide all types of information; hence, the outcome of any particular situation is difficult to predict from thermodynamic information alone Thermodynamics does for a biochemist what a contour map without roads would do for a motorist. It tells him how far above or below him his destination lies, but it alone does not tell him whether there is a road he can follow—it tells him to where he cannot coast Many reactions with negative ΔF do not proceed at a measurable rate, for example, gasoline is quite stable at room temperature in the presence of oxygen although its ΔF of oxidation is a large negative number A negative ΔF is thus necessary for a reaction to occur but is not sufficient to predict whether it will occur In other words, ΔF and the rate of a reaction are not related This is because a molecule must obtain a certain amount of energy (activation energy) before it can react, independent of the possibility that the entire reaction may release energy. Enzymes are necessary to make biological reactions proceed at measurable rates They do so by finding a pathway of lower elevation on the contour map The fact that energy-rich compounds may be unreactive is very important because it means that the path of metabolism is not inflexibly one that yields the most energy at each step, but instead can lead to accumulation of compounds of high free energy content.

B. Free Energy of Formation

Free energy values usually are tabulated as standard free energies of formation of the compounds This quantity is defined as the free energy

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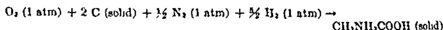
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B. Free Energy of Formation

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change of the reaction in which the compound in its standard state is formed from the elements which compose it, in their standard states at the specified temperature For example, the standard free energy of formation of solid glycine is −88,610 cal. This is the free energy change for the reaction.



$$O_2\ (1\ \text{atm}) + 2\ C\ (\text{solid}) + \tfrac{1}{2}\ N_2\ (1\ \text{atm}) + \tfrac{5}{2}\ H_2\ (1\ \text{atm}) \rightarrow CH_2NH_2COOH\ (\text{solid})$$

The free energies of formation of the elements are by this definition equal to zero. Tables of free energies and illustrations of the methods of calculation are available (*7–9*) Methods of determining ΔF will be presented in the remainder of this section

C Dependence of ΔF on Concentration

The free energy of a substance depends on concentration. Quantitatively, if C_1 and C_2 are two concentrations of a substance then F at C_1 is related to F at C_2 by the free energy change of the dilution. This work of dilution from C_1 to C_2 is equal to

$$RT \ln C_2/C_1 \tag{3}$$

where R is the gas constant (1.987 cal/mole/degree), T is the absolute temperature, and ln is logarithm to the base e. Rigorously, activities should be used in place of concentrations. (This refinement is not commonly used in biological work because the data are not sufficiently accurate and the activities are seldom known) For example, F of 10^{-7} M H^+ is equal to F of 1 M H^+ (defined as equal to 0) plus ΔF of the reaction *in which* 1 *mole of* 1 M H^+ *is diluted to* 1 *mole of* 10^{-7} M H^+. At 37°,

$$H^+\ (1\ M) \rightarrow H^+\ (10^{-7}\ M)$$
$$\Delta F = 1410 \log 10^{-7}/1 = -9870 \text{ cal}$$

This means that 9870 cal would have to be used to compress 1 mole of H^+ from 10^7 liters into 1 liter of water (assuming maximal efficiency)

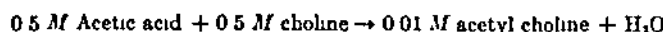
The logarithmic relation (Eq. 3) between ΔF and the concentration change has been presented without proof, but it may seem more reasonable if one considers the work required to compress a given amount of gas into successively smaller volumes, for example, from 100 ml to 10 ml and then from 10 ml to 1 ml. Although the volume change in the second step is only $\frac{1}{10}$ that of the first, the total *efforts are similar since the* pressure becomes increasingly great as the volume decreases; and *in fact* the amount of work is theoretically the same in the two steps *The* work would seem proportional to the per cent change in volume, and *a* logarithmic relation satisfies this requirement

Sometimes one is given the free energy of a component in the solid form and desires its free energy in solution (*10*). In order to calculate this quantity one separates the reaction into two parts. First, one uses the fact that F of a substance in saturated solution is the same as that of the solid, because the phases are in equilibrium. Second, by use of the solubility plus Eq. (3) to determine ΔF of dilution one calculates F in a solution of the desired concentration. For example, to calculate the ΔF^0 of formation of 1 M L-tyrosine (aq) one notes that ΔF^0 of formation of solid L-tyrosine is $-96{,}100$ cal/mole and its solubility is 0.0025 M. Therefore ΔF^0 of formation of 0.0025 M L-tyrosine is also $-96{,}100$ cal, and that of 1 M solution is $-96{,}100 + RT \ln 1/0.0025 = -92{,}500$ cal.

As mentioned previously, ΔF of a reaction depends on the concentrations of reactants and products, and it commonly differs from ΔF^0 by several thousand calories. The relation is easily derived from Eq. (3) by summation of free energy of products minus reactants.

$$\Delta F = \Delta F^0 + RT \ln (C)(D)/(A)(B) \tag{4}$$

To give an example of the application of Eq. (4), if one wished to synthesize acetylcholine, one might ask whether even a small concentration, let us say 0.01 M, of acetylcholine could be formed at 37° in the presence of 0.5 M choline and 0.5 M acetic acid. One determines ΔF for the reaction


0.5 M Acetic acid + 0.5 M choline → 0.01 M acetyl choline + H_2O

The ΔF^0 for this reaction is given as +3100 cal (*11*), and hence one calculates from Eq. (4)

$$\Delta F = +3100 + 1.987 \times 310 \times 2.3 \log \frac{(0.01)(1.0)}{(0.5)(0.5)}$$
$$\Delta F = 3100 - 1420(1.4)$$
$$\Delta F = +1100 \text{ cal}$$

Therefore 0.01 M acetyl choline will not be formed because ΔF is positive.

Note that the concentration of water is taken as unity. This means that water as the pure liquid rather than 1 M concentration of water is taken as the standard state. It is convenient to give liquid water the value unity rather than 55.5, which is the actual molar concentration of water in dilute aqueous solutions. However, one must always use the same convention for water as was used in defining ΔF^0, because a difference of 2500 cal (equal to $RT \ln 55.5$) exists at 37° in the ΔF^0 values calculated by the two methods. Liquid water is commonly assigned the value 1.0 in calculations involving the equilibrium constant. for example, the constant for the hydrolysis of acetylcholine is written

$$K = \frac{(\text{acetic acid})(\text{choline})}{(\text{acetylcholine})}$$

in some cases high concentrations of other materials, such as alcohol or glycerol, are present. The concentration of water is no longer 55.5 M, and the actual concentration must enter into free energy calculations (12, 13).

Equation (4) becomes the definition of ΔF^0 if all reactants and products are at unit concentration or are present as pure substances (i.e., are in their standard states). Then $\ln (C)(D)/(A)(B) = 0$ and $\Delta F = \Delta F^0 + 0$. Therefore, ΔF^0 is the free energy change when all reactants are in their standard states. In biochemistry, it is convenient to know ΔF when all reactants are in their standard states, except that pH is 7.0; this ΔF is called $\Delta F'$.

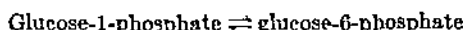
D. The Relation between ΔF^0 and the Equilibrium Constant

At equilibrium $\Delta F = 0$. Also, $(C)(D)/(A)(B) = K$, the equilibrium constant. Therefore, Eq. (4) becomes

$$0 = \Delta F^0 + RT \ln K,$$
$$\Delta F^0 = -RT \ln K \tag{5}$$

If ΔF^0 is known, K can easily be calculated from this equation. Conversely, one very useful way of determining ΔF^0 is to measure the equilibrium concentrations of reactants and products to determine K. This method is most valuable for reactions with ΔF^0 between plus and minus 3000 cal, because it is very difficult to measure the minute amounts of compounds present at equilibrium in reactions with extreme equilibrium constants. Radioactive compounds permit determinations over a greater range of ΔF^0 (13, 14).

Equilibrium measurements have been used to determine ΔF^0 for reactions such as hydrolyses and rearrangements. For example, at equilibrium the reaction


Glucose-1-phosphate ⇌ glucose-6-phosphate

gave 95% glucose-6-phosphate. Therefore $K = 19$ and $\Delta F^0 = -1800$ cal. There are numerous recent applications which provide excellent examples of this method of experimentation and calculation (15, 16a).

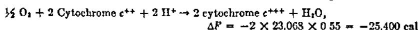
Equilibrium (as calculated from an equilibrium constant) is not often attained in living systems. For example, knowing the concentration of glucose-1-phosphate and the above constant does not permit one to calculate the concentration of glucose-6-phosphate in a living cell. The usual situation is one of a steady state in which concentrations are determined by relative rates of reactions which produce and remove compounds [see, for example, ref. (16b)]. New applications of thermodynamics are being developed to deal with these steady state systems (3, 17, 18).

E THE RELATION BETWEEN ΔF AND ELECTROMOTIVE FORCE

It is possible to obtain work from many reactions by forming an electrical cell in which the reaction occurs The familiar storage battery and dry cell are examples. In many cases it is possible to measure the maximum work available from the reaction quite precisely by measuring the voltage (electromotive force, E) of the cell E is proportional to the maximum work per electron transferred, and so it is related to ΔF by the equation

$$\Delta F = -nFE \qquad (6)$$

In this equation n is the number of electrons transferred according to the chemical equation as written and F is the Faraday constant (23,068 cal per volt equivalent) For example, for the oxidation of cytochrome c^{++} by O_2, $E = 0.55$ volt under standard conditions, with the exception that $H^+ = 10^{-7}\ M$ This E at pH 7 and other conditions standard is designated E_0'. Since two electrons are involved in the equation

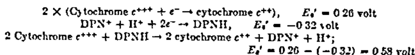


$$\tfrac{1}{2}\,O_2 + 2\ \text{Cytochrome}\ c^{++} + 2\ H^+ \rightarrow 2\ \text{cytochrome}\ c^{+++} + H_2O,$$
$$\Delta F = -2 \times 23{,}068 \times 0.55 = -25{,}400\ \text{cal}$$

E is positive if the reaction tends to proceed as written

Determination of ΔF by measurements of electromotive force are generally limited to oxidation-reduction reactions, but for these it provides a very valuable method Some oxidation-reduction reactions, e g. oxidation of —SH groups, are not reversible, only E of reversible reactions are useful for determining ΔF because E is a measure of maximum work only if the reaction is reversible This fact as well as many experimental details must be considered if one is to obtain reliable results (*19, 20*)

Many data on the energy changes in biological reactions have been recorded as E_0' of half reactions (*9, 21*) A half reaction is an equation in which a reducible compound takes up one or more electrons (as would occur at an electrode) Then hydrogen ions and water are added to balance the equation (see examples below) If any half reaction is subtracted from another involving the same number of electrons, e^-, one obtains a whole reaction (*22*) For example·



$$2 \times (\text{Cytochrome}\ c^{+++} + e^- \rightarrow \text{cytochrome}\ c^{++}), \quad E_0' = 0.26\ \text{volt}$$
$$DPN^+ + H^+ + 2e^- \rightarrow DPNH, \quad E_0' = -0.32\ \text{volt}$$
$$2\ \text{Cytochrome}\ c^{+++} + DPNH \rightarrow 2\ \text{cytochrome}\ c^{++} + DPN^+ + H^+;$$
$$E_0' = 0.26 - (-0.32) = 0.58\ \text{volt}$$

Note that one simply subtracts the E_0' values for the half reactions and does not take notice of the fact that the first reaction was multiplied by two The corresponding $\Delta F = -26{,}800$ cal, from Eq. (6).

Half reactions do not occur by themselves They are simply a convenient way of recording a great deal of information For instance, it is much more convenient to record 200 half reactions than 19,900 complete reactions, which could be made from them.

Unfortunately, there are two conventions for recording E of half reactions In chemistry, reactions are written as oxidations and the more easily oxidized compounds are given more positive E values, e g ,



$$Na \rightarrow Na^+ + e^-, \qquad E_0 = +2\ 7 \text{ volt}$$

In biochemistry, reactions are written as reductions, and compounds that are more easily reduced are given positive values; for example, at pH 7,



$$O_2 + 4\ H^+ + 4e^- \rightarrow 2\ H_2O, \qquad E_0' = +0\ 8 \text{ volt}$$

One system is the opposite of the other. In both cases, the greater the tendency to proceed as written (i e , the farther from equilibrium), the more positive the value of E. There is no difficulty if one system only is used, and it is helpful to remember the sign of some familiar reaction like the above for a landmark Occasionally one finds published values of E, or of ΔF, with an incorrect sign

Sometimes ΔF of a half reaction is reported If one finds this confusing, it may be useful to think of this ΔF as that of the reaction in an electrical cell with the other electrode having zero potential (e g H_2 electrode)

The dependence of E on concentration is easily obtained by substituting $-nFE$ for ΔF in the similar equation for ΔF [Eq (4)];

$$E = E_0 - \frac{RT}{nF} \ln \frac{(C)(D)}{(A)(B)}$$

F. The Determination of ΔF from Thermodynamic Data

Another method of determining ΔF is to make use of measurements of the heat released by a reaction and of the heat capacities of the reactants and products This method utilizes Eq (2). The heat released at constant temperature and pressure, ΔH, can be measured by calorimetry *(23, 24)* The entropy, ΔS, can be obtained from measurements of heat absorbed per degree by each compound separately at temperatures ranging from the temperature of the reaction to as low a temperature as possible; or other methods of calculating ΔS can be used *(25)* (see Section V) Such determinations are difficult to make because special techniques are required, and the quantities must be determined with great precision ($\pm$ 100 cal) to be useful, for if one wishes to calculate ΔF of some reaction (a few thousand calories) from ΔH data, then $\pm$ 100 cal is a very appreciable error (10%).

Factors for computing changes in ΔF° of formation for various structural chemical changes are available (*7, 26*). For example, one calculates $-52{,}780$ cal and $-56{,}640$ cal for modifications starting from alanine and tyrosine respectively, for ΔF° of formation of phenylalanine There is considerable uncertainty in such a calculation. It is more useful to have a value for ΔH and to estimate ΔS from structural factors in order to calculate ΔF

G Combination of Equations

For some reactions ΔF can be determined by the preceding methods, but other reactions have not been studied directly. By combining two or more reactions and their ΔF values it is possible to obtain ΔF for a third reaction that would be difficult or impossible to study directly An important example of this is the ΔF of hydrolysis of the terminal phosphate of ATP. No direct means of obtaining this quantity has yet been devised; therefore, indirect methods have been used As an example of an indirect determination, we may illustrate with one calculation of this quantity (*14, 14a*).

Equilibrium measurements of the hexokinase reaction



$$\text{Glucose} + \text{ATP} \rightleftharpoons \text{glucose-6-P} + \text{ADP}$$

and for the hydrolysis of glucose-6-P



$$\text{Glucose-6-P} + H_2O \rightleftharpoons \text{glucose} + PO_4$$

gave $\Delta F'$ values of -4700 cal/mole and -3100 cal/mole respectively (corrected to pH 7 0 and 30°) The sum of these reactions is



$$\text{ATP} + H_2O \rightleftharpoons \text{ADP} + PO_4$$

and the sum of the $\Delta F'$ values gives $\Delta F' = -7800$ cal

More involved calculations, requiring combination of numerous equations and their ΔF values, have been used to determine ΔF of hydrolysis of ATP The results gave only rough approximations [see references in (*27, 28*)], and the principal merit of calling attention to these calculations is to point out their uncertainty.

It is important to stress that such calculations require many corrections which should be made in precise work, many of them not obvious, such as corrections for degree of ionization, complexes by metal ions, temperature, etc. These are best understood by working out examples (*7, 9, 10*)

II. Dependence of Free Energy on pH

The ΔF of a reaction can depend greatly on pH, as can be seen from Fig 1 (*29*). Such curves can be calculated from Eq (4) combined with

do not necessarily occur at a measurable rate, even though ΔF is negative. Third, the maximum heat that a reaction can evolve is not equal to the maximum possible useful work, i e., ΔH is not equal to ΔF.

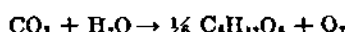
III. Sources of Energy

Energy flows through the living world commencing principally with absorption of sunlight by photosynthesizing organisms. It is stored as chemical energy, released by breakdown of compounds to do work and perform syntheses, and finally discarded as heat and products of low energy content. In the following sections some of the present knowledge of this energy flow will be used to indicate the wide application of free energy to various fields.

A. Photosynthesis

As is well known, almost all energy used by living organisms initially comes from sunlight Light is absorbed by plants and is transformed into chemical energy which appears as carbohydrate and other cell material The efficiency of conversion of light energy to chemical energy by field crops is estimated to be 2% at best (*30*) As much as 20% efficiency can be obtained under laboratory conditions, using algae or sections of leaves as plant material Under large scale conditions up to 6% efficiency has been found.

The terms "light energy" and "chemical energy" must be defined more carefully It will be recalled that light energy is present in units called photons A photon has a definite amount of energy, one quantum, equal to Planck's constant times the frequency of light The energy of one mole of red light (wavelength 700 mμ) is about 40,000 cal and of blue light (440 mμ) is about 64,000 cal A quantum of red light is just as useful for photosynthesis as a quantum of light of higher energy, the excess energy of which is lost as heat or fluorescence (*31*) Light of wavelength longer than about 700 mμ is not very effective for photosynthesis in plants, but in bacteria wavelengths as high as 900 mμ (30,000 cal) can be used (*32*) The chemical changes brought about actually result in the synthesis of new cell material; but a large part of this is carbohydrate, so that in general the chemical reaction is written



$$CO_2 + H_2O \rightarrow \frac{1}{6}\, C_6H_{12}O_6 + O_2$$

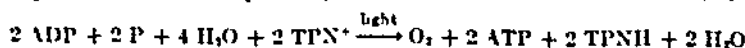
The ΔF of this reaction is +118,000 cal under physiological conditions The over-all reaction is usually measured by changes in gas pressure Actually the fixation of CO_2 involves an extremely complex reaction sequence (*33, 16b*)

The efficiency of the over-all process is determined from the CO_2 taken up per quantum absorbed—the quantum requirement. The seemingly simple problem of determining the quantum requirement is still the subject of vigorous debate The earliest measurements, indicating a quantum requirement of about 4, were made in 1923, and Warburg (*34*) recently obtained quantum requirements as low as 3; but many other workers have obtained values of about 8 to 10 (*31*, *35*, *36*) The free energy, +118,000 cal, is all eventually provided by the absorbed light; therefore, about 3 quanta of red light per $\frac{1}{6}$ glucose would be needed for a completely efficient reaction. Warburg's most recent results would indicate a very high efficiency, better than 90%, but a figure of 8 quanta would mean that the reaction is only about 35% efficient

Warburg (*34*) has secured evidence for reoxidation of about 70% of the reduced material formed after light absorption He suggests that a quantum requirement of one is achieved over short periods of illumination (fixation of one CO_2 into some intermediate and release of one O_2 per quantum), followed by reduction of $\frac{2}{3}$ or $\frac{3}{4}$ of the oxygen, giving an over-all quantum requirement of 3 or 4 quanta per O_2. Objections to Warburg's experiments have been raised (*36*) and it seems impossible to decide the matter at present Oxidation of reduced coenzyme or carbon-containing compounds would result in formation of energy-rich phosphate, which could be used by the usual channels (*37*) (see below)

Now that the photosynthetic process can be separated into stages, it would seem more pertinent to ask what are the efficiencies of the individual steps In particular, what is the efficiency of the initial conversion of light energy into the first stable chemical intermediate? Light is absorbed by one of a number of pigments and the energy is efficiently transferred to chlorophyll *a* (*35*) This energy can be used to produce O_2 from water and transfer hydrogen to other compounds, without CO_2 fixation Hill showed that a preparation of mashed leaves released O_2 and produced ferrous ions when illuminated in the presence of ferric salts (*37*) Other compounds also were able to serve as electron acceptors. The naturally occurring coenzyme TPN served as a hydrogen acceptor if the reduced TPN was rapidly reoxidized by a subsequent reaction (*33*). Two quanta (80,000 cal/mole) would be ample to reduce one TPN with production of $\frac{1}{2}$ O_2 (see below), however, the measured quantum requirement of the Hill reaction was 11 (*37*).

Reduction of TPN and phosphorylation of ADP have been achieved in preparations of chloroplasts (*38*) The over-all reaction is written



$$2\ ADP + 2\ P + 4\ H_2O + 2\ TPN^+ \xrightarrow{\text{light}} O_2 + 2\ ATP + 2\ TPNH + 2\ H_2O$$

Presumably in such a system one is close to the primary steps of con-

The compound to which starting materials are degraded is sometimes assumed to be acetate (2-carbon units) or pyruvate. Measured efficiencies of about 5% for glucose and 25% for acetate have been found in microorganisms, and one calculation for the rat has yielded an efficiency of glucose assimilation of about 20% (*48*).

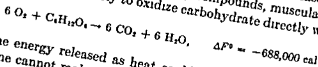
Considerable evidence supports the view that what limits assimilation is not energy but rather a supply of molecular building blocks suitable for formation of larger molecules (*46, 47*). The same reactions which provide energy also form building blocks; and energy may be released that is not utilizable because not enough energy-accepting molecules are available. Thus, the amount of material assimilated by *Escherichia coli* from succinate or fumarate is the same although the former furnishes more free energy on oxidation. In many cases, one mole of substrate produces a whole number of moles of CO_2; for example, one CO_2 is produced per acetate by *Prototheca zopfi*, and three CO_2 are produced from two acetate by *E. coli*. This may be considered evidence for control of assimilation by the necessity of certain reactions occurring rather than by energy supply. The reason for the apparently low machine efficiency of *Chlorella* on glucose (*49*), about 4%, is clear when it is noted that in order to achieve this efficiency about 60% of the carbon must be assimilated. Certainly the losses of CO_2 necessary during formation of the building blocks must determine the balance sheet. Energy must be available in excess.

IV. Release of Energy

A. Controlled Energy Release

There are few areas of biochemistry to which thermodynamics has been more applied than to degradation of carbohydrates and fats. This is the chemistry of energy release. The stored energy is released to permit growth, synthesis of the many necessary compounds, muscular work, etc.

It is not sufficient merely to oxidize carbohydrate directly with oxygen

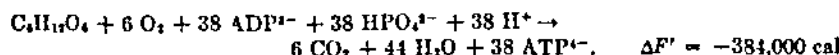


$$6\ O_2 + C_6H_{12}O_6 \rightarrow 6\ CO_2 + 6\ H_2O, \qquad \Delta F^0 = -688{,}000 \text{ cal}$$

because the energy released as heat could not be used by the cells. By analogy, one cannot make an automobile run by putting a match to the gasoline; rather, a mechanism must be present to control the gradual release of energy in some usable form.

The key to how locked-up chemical energy of starch and fat is made usable in a controlled way is found in the chemistry of phosphate compounds (*50–52*). In 1905 Harden and Young found that phosphate is involved in the metabolism of glucose, and as an over-all reaction one

may now write



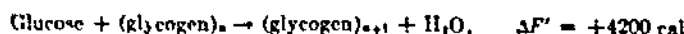
$$C_6H_{12}O_6 + 6\ O_2 + 38\ ADP^{3-} + 38\ HPO_4{}^{2-} + 38\ H^+ \rightarrow 6\ CO_2 + 44\ H_2O + 38\ ATP^{4-}, \quad \Delta F' = -384{,}000 \text{ cal}$$

It can be seen that not all of the 710,000 cal free energy available from oxidation of glucose under physiological conditions is released About 460,000 cal or 65% is stored in ATP (assuming ΔF of hydrolysis of the terminal phosphate of ATP *in vivo* is about −12,000 cal, due to *the low phosphate level and ATP/ADP ratio greater than 1) (52, 53).* Thus, the principal method of conversion of chemical energy to useful processes, as we understand it at present, is as follows large molecules [illegible]

can then be doled out to energy-requiring reactions In succeeding paragraphs, some comments on the mechanism of stepwise production of ATP will be presented However, the subject is discussed in detail in other chapters of this book and in recent reviews (*9*)

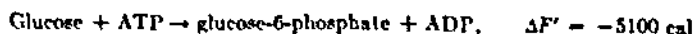
B Coupling of Reactions

Many reactions with an apparently positive ΔF occur in living cells *Examples include syntheses of all sorts including formation of urea, proteins, polysaccharides, and fats (9) Such a reaction cannot proceed* alone; for the same laws of thermodynamics govern living and nonliving systems, and state that only reactions with negative ΔF are spontaneous. Some other reaction must provide energy to drive a reaction with positive ΔF There must be some mechanism for coupling the energy-supplying (exergonic) and energy-utilizing (endergonic) reactions. A most important general principle is that energy can only be transferred from one reaction to another by a chemical compound common to both reactions (*53, 54*) The most common source of energy is ATP which acts as a sort of messenger boy and delivers energy to endergonic reactions ATP can act by phosphorylating a reactant; this stores sufficient energy to make the free energy change of the subsequent reaction negative when it occurs with release of phosphate We may take as an example the formation of glycogen from glucose

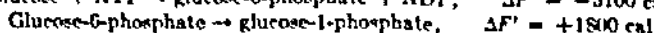


$$\text{Glucose} + (\text{glycogen})_n \rightarrow (\text{glycogen})_{n+1} + H_2O, \quad \Delta F' = +4200 \text{ cal}$$

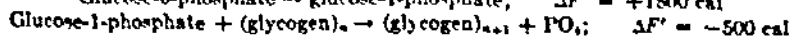
The required energy is provided as follows



$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}, \quad \Delta F' = -5100 \text{ cal}$$



$$\text{Glucose-6-phosphate} \rightarrow \text{glucose-1-phosphate}, \quad \Delta F' = +1800 \text{ cal}$$



$$\text{Glucose-1-phosphate} + (\text{glycogen})_n \rightarrow (\text{glycogen})_{n+1} + PO_4; \quad \Delta F' = -500 \text{ cal}$$

The over-all reaction is the sum of the above

$$\text{Glucose} + (\text{glycogen})_n + \text{ATP} \rightarrow (\text{glycogen})_{n+1} + \text{ADP} + \text{PO}_4$$

and has a negative $\Delta F'$ under physiological conditions; $\Delta F' = -3800$

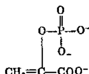
It will be noted that the conversion of glucose-6-phosphate to gluc 1-phosphate requires energy under standard conditions However, actually must be negative, and this is accomplished by a build-up glucose-6-phosphate by the previous reaction and a removal of glucose- phosphate by the subsequent reaction. This is the second method b which reactions with an apparently positive $\Delta F'$ are made to proceed Coupled reactions occur which change the free energy by increasing the concentration of a reactant or decreasing that of a product (53) Actually, the problem is an artificial one because the standard free energy is an arbitrary quantity, defined under conditions which do not correspond to actual conditions in the cell Therefore, ΔF of the reaction, which is the important quantity, must really be negative and it is merely $\Delta F'$ that is positive

C. High-Energy Phosphate

The energy released by hydrolysis of ATP is considerably greater than the amount released by hydrolysis of glycerophosphates, ethyl phosphate, or other esters, all of which release about 3000 cal per mole (28, 29). The latter are termed "low-energy phosphate compounds" (*−ph*). ATP is termed a "high-energy phosphate compound" (*~ph*). The two terminal phosphate bonds, the energies of hydrolysis of which are responsible for the designation "high-energy," are actually acid anhydride bonds made from two phosphoric acids Such bonds, like those of acetic anhydride, useful for acylations in organic chemistry, are termed "high energy bonds" in biochemistry. Mixed anhydrides of carboxylic and phosphoric acid, such as acetyl phosphate and 1,3-diphosphoglyceric acid, and anhydrides of phosphoric acid (ATP, ADP and various coenzymes containing pyrophosphate such as coenzyme I) are also "high-energy" compounds. Only the two terminal phosphates of ATP and one phosphate of diphosphoglyceric acid are *~ph*. Other types of high-energy compounds are found in biological systems One type is represented by phosphocreatine (and phosphoarginine)

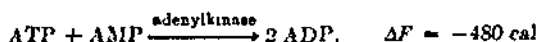
```
    O⁻        N—H
    |         ‖
O═P—N——C—N—CH₂—COO⁻
    |    |         |
    O⁻   H        CH₃
```

Others are acyl coenzyme A derivatives and phosphoenolpyruvate



The $\Delta F'$ of hydrolysis of any $\sim ph$ compound has not yet been determined directly Various calculations have been made, and the more recent ones agree fairly well in indicating $\Delta F'$ of ATP hydrolysis to be about -7000 to -8000 cal (*27*)

By equilibrium measurements the differences between free energies of hydrolysis of various $\sim ph$ compounds have been determined. The two $\sim ph$ bonds of ATP are of about equal strength (*65*). At pH 7.4

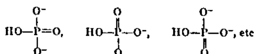


$$\text{ATP} + \text{AMP} \xrightarrow{\text{adenylkinase}} 2\ \text{ADP}, \qquad \Delta F = -480 \text{ cal}$$

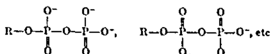
The equilibrium between phosphocreatine and ATP shows that the former compound has a $\Delta F'$ of hydrolysis 3,200 cal more negative than that of ATP (*29*); however, the value is very dependent on pH (see Fig 1) and on Mg^{++} (*66*). The $\Delta F'$ of hydrolysis of 1,3-diphosphoglyceric acid, phosphoenol pyruvate and acetyl phosphate are all of somewhat higher energy, about $-12{,}000$ cal. Summaries of such values have been published (*9*, *29*).

The hydrolysis of low-energy phosphate compounds has been studied by equilibrium methods and the energy is found to be in the range of $-2{,}000$ to $-4{,}000$ cal per mole (*28*) The high concentration of water as compared to phosphate is largely responsible for the negative value of this $\Delta F'$

Three features of the structure of $\sim ph$ compounds are principally responsible for their high energy of hydrolysis One is the relatively higher resonance stabilization after hydrolysis Inorganic phosphate possesses a number of resonance structures of similar energy·



In a pyrophosphate compound there are fewer important resonance forms per phosphate



Since fewer resonance forms are possible in the latter compound t internal energy is lowered less, and the energy difference upon hydroly is greater than in ordinary phosphate esters, in which the phosphate h about the same number of resonance forms as in phosphate ion

A second structural feature which lends pyrophosphate its high energy character is the nearness of many charged groups. Energy is required to overcome the electrostatic repulsion of like charges, and is released when the groups are separated The third source of energy is found in the ΔF of neutralization of acid groups produced upon hydrolysis.

The high energy of hydrolysis of acyl phosphates and of phospho creatine depend to some extent on similar resonance forces, but phos phoenolpyruvate, the fourth type of high-energy phosphate compound, has a high energy of hydrolysis because pyruvate is held by the phosphat group in the much less stable enol form $CH_2{=}COH{-}COOH$ rathe than the usual keto form $CH_3{-}CO{-}COOH$. Hydrolysis provides the ordinary energy of a phosphate ester bond plus the energy of conversion of enol to keto form. More complete discussions of the structure of ~ph compounds are available (*28, 67*).

Some other compounds which provide an extraordinarily large amount of energy (about −8000 cal) upon hydrolysis are the compounds of coenzyme A (CoA) acetyl CoA, butyryl CoA, succinyl CoA, etc. (see other chapters of this book) The coenzyme possesses a terminal —SH group to which the acyl group is attached in such a way as to yield this large ΔF upon hydrolysis. Equilibrium data indicate that the bonds formed between amino acids and nucleotides or ribonucleic acid, and also the phosphodiester bonds in ribo- and deoxyribonucleic acids, must have $\Delta F'$ of hydrolysis of about −8000 cal/mole (see Chapters 17 and 19, Volume II)

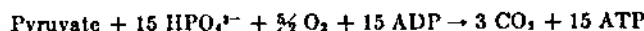
D. Production of High-Energy Phosphate

The reactions by which high-energy phosphate is produced, especially by metabolism of glucose, have been intensively studied in recent years and a large body of thermodynamic data has accumulated (*9*). Since these reactions will be discussed in detail in subsequent chapters, little will be done here except to present efficiencies of reactions

The conversion of one glucose to two lactates in mammalian tissue leads to the formation of two molecules of each of two high-energy phosphate compounds· 1,3-diphosphoglyceric acid and phosphoenolpyruvate These compounds can transfer phosphate to ADP and form a total of four molecules of ATP. However, two molecules of ATP are required to make the reaction proceed, so the net gain is only two ATP. Of the approximately 47,000 cal available from the reaction, 24,000 cal are

conserved as $\sim ph$ (51%), assuming ΔF of hydrolysis of the terminal phosphate of ATP is $-12{,}000$ cal per mole *in vivo*.

Under aerobic conditions two pyruvates and two molecules of DPNH are formed instead of two lactates. Oxidation of the two coenzyme molecules ($\Delta F = -52{,}000$ cal per mole) probably yields six more $\sim ph$. Oxidation of two pyruvates, via the citric acid cycle, to CO_2 and H_2O yields about 30 more $\sim ph$, formed in stepwise fashion

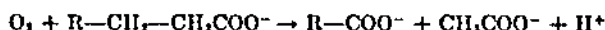


$$\text{Pyruvate} + 15\ HPO_4^{2-} + \tfrac{5}{2}\ O_2 + 15\ \text{ADP} \rightarrow 3\ CO_2 + 15\ \text{ATP}$$

These 30 ATP conserve about 360,000 cal of the 546,000 cal available from direct oxidation of two pyruvate *in vivo* (66% efficiency).

Each transfer of two electrons from a substrate in the tricarboxylic acid cycle to oxygen yields two to four $\sim ph$. It seems likely that in the process of electron transport via a chain of carriers (coenzymes, flavoproteins, cytochromes, and cytochrome oxidase) various steps are coupled with production of one $\sim ph$ each. Unfortunately, most of the details of this process are not yet available.

In the oxidative removal of two carbons from a fatty acid one FAD and one DPN are reduced and one acetyl CoA is formed. Five $\sim ph$ probably are formed from the oxidation of the coenzymes by O_2, plus one from acetyl CoA. These $\sim ph$ contain 72,000 cal of the approximately 95,000 cal available, under physiological conditions, from the reaction



$$O_2 + R{-}CH_2{-}CH_2COO^- \rightarrow R{-}COO^- + CH_3COO^- + H^+$$

The efficiency is thus about 76% under these conditions.

E. Storage of High-Energy Phosphate

There are approximately 5×10^{-6} moles of ATP per gram of skeletal muscle, and this is capable of storing only a minute amount of energy (0.07 cal per gram of tissue). The prime function of another compound in mammalian muscle, phosphocreatine, seems to be to supplement this store of $\sim ph$. It is present to the extent of 20×10^{-6} moles per gram [illegible]

of tissue) for muscle contraction. Phosphoarginine takes the place of phosphocreatine in some invertebrates.

F. Utilization of Energy

Considerable information has been accumulated concerning mechanisms and energetics of synthetic reactions; however, much more remains

to be gathered. ATP is involved in many of the fairly well understood reactions (*9, 16b, 52*). Usually only a fraction of the energy of ATP is used, for example, about 3000 cal are required for formation of an amide or ester bond yet one ~*ph* having a ΔF of about 12,000 cal is actually used. Thus, such a reaction is only about 25% efficient, and the bulk of the energy of the high-energy compound is given off as heat.

Metabolic energy is used via ATP for a variety of other processes including muscle contraction (*58, 59*), nerve conduction and electrical discharge (*60*), light production (*61*), and transport of material against a concentration gradient (*62, 63*).

Efficiency of utilization of the energy for muscle contraction appears to be of the order of 25% for the over-all process and 45% for the initial process (*64*) The over-all process in nerve conduction appears to be at least 10% efficient and the primary process may use as much as 45% of the energy (*65*). Forty per cent of the chemical energy appears as electrical energy in the discharge of the electric eel (*66*). Gastric secretion may possibly be nearly 100% efficient (*67*). Only 8% of the metabolism of a red blood cell is required for the transport of ions (*68*). Other references on energetics of osmotic work (*69*) and muscle contraction (*70-72*) are available. Efficiency values seem quite high as compared to efficiencies of mechanical engines. These interesting subjects seem to be beyond the purpose of this chapter which is primarily to provide an introduction to applications of thermodynamics elsewhere

V. Entropy

A. Introduction

Entropy is important in the study of enzyme reactions because it contributes to the free energy of the reaction, determines the temperature dependence of this free energy change and often gives clues as to the reaction mechanism. [For a good review see reference (*25*)] The specific uses of entropy values will become more evident in the discussion to follow.

[illegible] sequences

means death whereas life is characterized by order or negative entropy. The organism maintains this order or life by consuming highly ordered complex organic compounds. These compounds are broken down to CO_2 and water with the simultaneous disorganizations of molecular structure For example, respiration causes the breakage of many carbon-carbon bonds to form simpler molecules. Carbon-carbon stretching motions are

thus converted to translational motions. As will be apparent in later discussion, such a process will be accompanied by an increase in entropy.

The entropy value is, at any given temperature, a measure of the system's degree of randomness or disorientation Processes tend to proceed toward systems of greater randomness For example, if balls neatly lined up in a basket are shaken, the result will be a random distribution of higher probability than the first. There has been no energy change between initial and final states but a process has taken place.

Entropy is defined by the equation,

$$S = k \ln W \tag{8}$$

where W is the number of ways of arranging the molecules in their various energy levels without changing the energy of the system (*74*). It should be remembered that all energies are quantized so that even translational energies are only possible in discrete levels and not as a continuum of translational energies W is a number with a value of one or greater which becomes larger as the complexity of the system increases. For example, if two molecules are in the same energy level, W is 1. If two molecules a and b are in energy levels 1 and 2, the distribution could be a in 1 and b in 2 or b in 1 and a in 2, so W is 2. Distributions of equal energy for three molecules a, b, and c, with two molecules in level 1 and one in level 2 would be a-2, b-1 and c-1; a-1, b-2 and c-1 or a-1, b-1 and c-2, therefore W would be 3

When the values of the energy levels are known, it is possible to calculate the distribution of molecules among these energy levels, assuming the distribution obeys the Boltzmann law. Statistical methods allow a calculation of W and the entropy In this manner it is possible to use statistics to calculate entropies of molecules from a knowledge of molecular energy levels (*75*, *76*).

As thermal energy is added to a system the entropy increases. This is because at absolute zero all the molecules are in their lowest energy levels There is only one way to arrange the system with all the molecules in the lowest energy level W equals one and the entropy, S, is zero At higher temperatures the molecules become distributed among the many higher energy levels so that W, the number of ways of arranging molecules, increases and the entropy increases.

The absolute entropy of a compound depends upon the amount, the temperature, and the physical state of the compound. Entropies are commonly expressed for a mole of the compound in its standard state at 25°. This procedure will be followed here unless other conditions are specified. Entropy units (eu) are cal/mole/°C. One entropy unit (1 eu) will contribute about −0 3 Kcal [−0 298 Kcal at 298°] to the free energy.

Changes in entropy are independent of pathway and only depenc upon the initial and final states of the system. The first four Sections (A–D) of this discussion will be concerned with the entropies of compounds, the last four Sections (E–H) will be concerned with the differences in entropy between the initial and final states of a process.

Types of energies that contribute greatly to the energy of the molecule contribute little to the entropy and vice versa. The value W may be approximated as a product of W [electronic], W [vibrational], W [rotational], and W [translational] so that each of these entropy terms may b discussed separately The widely spaced electronic levels are quite important in determining the energy of a molecule but have no effect on

TABLE I

ENTROPY (77) OF n-PROPANE AT 231 04°K

Electronic	None
Vibrational	1 05
Rotational	23 38
Translational	36 02
	60.45

entropy The electronic levels are so far apart in energy that there random distribution of molecules All are in the lowest electronic at normal temperatures so that W [electronic] is unity and S [electi is zero Translational energy levels are the other extreme. These levels that lie close together do not contribute much to the energy of molecule The molecules are widely distributed among these availe levels, the probability, W [trans], for such a random distribution is h and the S [trans] is high The importance of entropy terms for modera sized molecules increases in the series electronic, vibrational, rotationa and translational The magnitude of the terms shown in Table I com prising the entropy of n-propane is instructive

In macromolecules the greater number of vibrational frequencies makes the entropy of vibration quite important although the entropy per vibrational degree of freedom is small compared to translation. We see then that a knowledge of the entropy will give information primarily concerning the translational and rotational states of the molecule and also the vibrational states for large molecules

B PHYSICAL STATE AND ENTROPY

The physical state of the compound contributes greatly to the entropy of a compound. A gas at the boiling point has many more

degrees of freedom than a liquid at the boiling point and so the gas has a higher entropy (for example ΔS of vaporization of water is 26 eu). Similarly liquids have much higher entropies than solids (entropy of melting of ice is 5.3 eu)

In solution the primary effects on entropy are solvation by solvent and hydrogen bonding When the compound is dissolved in a polar solvent the solvation of ions and polar groups is a large factor in reducing the entropy of the system The solvent molecules held rigidly around the solute are restricted in translation and rotation so the entropy is low.

Small ions or multiply charged ions are more highly solvated than large ions or singly charged ions. In addition anions are more highly solvated in water than are cations (*78*) The entropy of the solvent is assumed for convenience to be normal and the entropy of solvation is assigned to the ion This causes the entropy values of small ions to be negative (*79, 80*) Values for entropies of ions in solution have been determined for various inorganic (*81, 82*) and organic ions (*83*).

In liquids where hydrogen bonding occurs the entropy is greatly reduced Association between molecules restricts rotation and translation and decreases the entropy Both *n*-butanol and diethyl ether have the same empirical formula but the entropy of the alcohol is 54 5 eu compared to 60 5 eu for ether Similarly, the entropy of *n*-butyric acid, 54 1 eu, is much less than that of ethyl acetate, 62 0 eu

C Structure and Entropy

From the structure of a molecule it is possible to gain much information about the relative entropy of the compound. One of the most important structural factors influencing entropy is size Both the number and the weight of the atoms comprising the molecule affect the entropy (*84*). Generally, large molecules have more degrees of freedom than small molecules and the energy levels are closer together The molecules are distributed among more energy levels and the entropy is higher The entropy of *n*-hexane is 70 6 eu whereas the entropy of *n*-hexyl bromide with a heavier atom substituted for hydrogen is 108 33 eu The entropy of naphthalene is 39 9 eu compared to 49 6 eu for anthracene which contains four more atoms [All values are from reference (*85*) unless otherwise specified]

Cyclic compounds are restricted in internal rotation and hence have less entropy than the corresponding open chain compounds The entropy of cyclohexane (l) is 49 2 eu whereas *n*-hexane (l) is 70 6 eu The entropy of cyclopentane (l) is 47 0 eu compared to 62 8 eu for *n*-pentane.

The entropy of hydrates are commonly increased over the parent compound by the entropy of ice. Water held as a hydrate is more like

ice than like liquid water. The entropy of liquid water is 16 75 eu whereas the entropy of ice extrapolated to 25 0° is 9 4 eu The entropy of asparagine hydrate with 51.4 eu is 9.4 eu greater than asparagine with 41.7 eu Similarly, $CaSO_4 \cdot 2H_2O$ with 46.4 eu is 10.45 eu per mole of water greater than anhydrous $CaSO_4$ with 25 5 eu. When the water is held tighter than a hydrate as in $Ca(OH)_2$ the difference is less than the value for ice. The entropy for $Ca(OH)_2$ is 17.4 eu and that of CaO, 9.5 eu, with a difference of 7 9 eu.

Symmetrical molecules have fewer rotational energy levels and hence a lower entropy. An N-fold symmetry reduces the entropy by $R \ln N$. In most biochemical compounds there is no symmetry so this factor will not concern us.

Resonance is primarily a correction to the electronic energy levels so that the resonance correction to the entropy of the compound is relatively small compared to the large ΔH correction (*86*). The small entropy correction is primarily because of the greater symmetry of benzene over that of the hypothetical cyclohexatriene. Resonance energies are almost always listed as ΔH values, the small entropy correction is commonly neglected.

D. Sources of Entropy Values

The most convenient sources of entropy values are the numerous tables giving the entropies of organic and inorganic compounds. Tables of entropy values will be found in various books (*7, 8, 85, 87, 88*). If the entropy of the compound cannot be found in one of these tables, it is possible to calculate the entropy from the standard free energy and standard enthalpy of the compound if these later values are known [cf Eq (2)].

It is quite often possible to estimate entropies from various empirical rules based on structural changes (*89*) Accurate methods have been devised by Parks and Huffman (*7*), and also by Anderson, Beyer, and Watson (*90*) The Parks and Huffman rules are based on the structural changes given in Table II from known entropies of normal paraffinic hydrocarbons If the entropy of the parent normal paraffinic hydrocarbon is not known it can be estimated by means of the following equations,

$$\begin{aligned} S^0_{298} &= 18\,0 + 5\,8\,n \text{ solid} \\ S^0_{298} &= 25\,0 + 7.7\,n \text{ liquid} \\ S^0_{298} &= 34\,0 + 10\,0\,n \text{ gas} \end{aligned} \tag{9}$$

where n stands for the number of carbon atoms The first term in the equations is called the basic molar entropy for that particular state and is used alone in conjunction with Table II For example the entropy of

TABLE II

THE CHANGES IN MOLAL ENTROPY AND FREE ENERGY ACCOMPANYING VARIOUS STRUCTURAL MODIFICATIONS AT 298.1°K*

Substitution or insertion	Change in Molal Entropy			Change in ΔF°_{298}
	Solid	Liquid	Gaseous	
(1) CH_2 into a hydrocarbon chain	5 8	7 7	10 0	1,080
(2) CH_3 for H attached to a main hydrocarbon chain	5 0?	3 2	5 0?	1,900
(3) C_2H_5 for H attached to a hydrocarbon chain	—	10 0	—	3,000
(4) CH_3 for H in a hydrocarbon ring	5 8	7 7	—	0?
(5) C_2H_5 for H in a hydrocarbon ring	11 6?	15 4	—	1,100
(6) Phenyl group for H attached to carbon	17 0	19 5	—	36,000
(7) Cyclohexane or cyclopentane ring for H attached to carbon	—	26 5	—	13,000
(8) Ethylenic double bond for a single bond	−2 7	−2 7	−2 7	Very irregular; mean about 20,000
(9) OH for H to form a monohydroxy, primary alcohol	0?	−1 5	13 0	−34,000
(10) OH for H to form a monohydroxy, secondary alcohol	0 5?	−4 0	9 0?	−37,000
(11) OH for H to form a monohydroxy, tertiary alcohol	0 5?	−6 0	7 0?	−41,000
(12) OH for H to form a phenol	0 0	0?	—	−41,000
(13) OH for H to form polyhydroxy compounds	0 5?	0 5?	—	
			primary OH	−34,000
			secondary OH	−37,000
(14) —O—linkage in a chain to form an ether	—	5 0	8 0	−20,000?
(15) O for 2H to form an aldehyde	1 0?	5 0?	7 3?	−23,000?
(16) O for 2H to form a ketone	1 0	0 5?	6 0	−30,000
(17) CO_2H for H to form a carboxylic acid	5 8	7 7	—	−83,200
(18) —C(=O)—O—group into a hydrocarbon chain to form an ester	—	13 2	21 0	−70,000
(19) NH_2 for H to form an amine	0 0	0 0	—	6,000
(20) NO_2 for H to form a nitro compound	7 0	8 0	—	7,000
(21) Cl for H	6 0	7 0?	9 0?	−1,600
(22) Br for H	7 5	9 0?	11 5?	4,500?
(23) I for H	9 0	11 0?	14 0?	10,000?
(24) Bivalent S for O	2 0?	2 0?	2 0?	36,000?

* Data taken from reference (7), p. 210.

liquid benzene at 25° is estimated as 19.5 [for a phenyl group from Table II] + 25 0 [basic molar entropy for liquid state] = 44.5 eu The difference between 44.5 eu and the observed value, 41.9 eu, is partly the result symmetry.

The difficult problem in estimating entropies occurs when the compound is in solution and it is necessary to correct for the entropy of solvation This is particularly a problem with ions. The entropy of solvated ions becomes more negative with increasing charge, Z, and less

TABLE III
STRUCTURAL CORRECTION VALUES, S_s, FOR THE COBBLE EQUATION

Double bond	3 5
Triple bond	4 5
Ring	14
Branched chain	3 0
≥3 Adj OH groups	16

TABLE IV
ENTROPY VALUES ESTIMATED BY THE COBBLE EQUATION

	Estimated	Observed
Glycine	38	41 1
D,L-leucine	50	50 8
L-tyrosine	62	59 0
L-cystine	56	55 0

negative with increasing radius, r Empirical equations (*91, 92*) for aqueous ions of the form

$$S = a - bZ^2/r \tag{10}$$

fit the data for many aqueous anions where a and b are structural constants For small or moderate sized ions the second term is larger than the first so that the entropy of these ions in solution is negative

Cobble (*93*) has solved the problem of the estimation of the entropies of neutral solutes by correcting for the molar volume, Vm, of the pure solute. The entropy of complex neutral solutes may be calculated by means of equation

$$S = 10 + \tfrac{3}{2} R \ln M + 9\,2\,N - S_s - 0\,22\,Vm \tag{11}$$

The symbol N is the number of skeletal atoms, M is the molecular weight, and S_s is a structural correction shown in Table III. Cobble has estimated by use of Eq (11) the entropy values given in Table IV.

The reader should be aware of the methods used to determine entropies experimentally. The classic method is to calculate the entropy from the heat absorbed by the compound as it warms from absolute zero to room temperature. These entropies are called "third law" entropies after the thermodynamic law stating that the entropy is zero at absolute zero

Entropies of gases may be determined by performing complete vibrational analyses of the infrared and Raman spectra in order to determine the rotational and vibrational energy levels and using statistical mechanics to calculate W and the entropy

E. CHANGE IN ENTROPY DURING REACTION

In the previous sections the entropy of compounds was discussed. In this section the discussion will be concerned with the difference in entropy between the products and reactants

Changes in entropies during reaction may be found by adding together the entropies of the products and subtracting the sum of the entropies of the reactants. They may also be determined from the temperature dependence of the free energy of the reaction; or by measuring both the heat of reaction and free energy of reaction and calculating the entropy by means of Eq (2)

Again it will be found that one of the most important factors contributing to the changes of entropies during reaction in aqueous solution is the entropy of solvation

The entropy changes during ionizations are particularly important to biochemists One might expect the entropy of ionization to be positive because the free ions have more translational and rotational degrees of freedom than the undissociated molecules But the solvation of the charged ions is a much more important factor causing the over-all entropy of ionization to be a large negative number. The entropy of ionization of a weak acid is about −22 eu (*94*, *95*); that of acetic acid is −22.1 eu and that of butyric acid is −21 4 eu (*96*) The decrease in entropy during acidic ionization of the Zwitterion form of amino acids is small [−7 to −10 eu] because the zwitterion itself is strongly solvated. The ionizations of acidic metmyoglobin (*97*, *98*) and acidic methemoglobin are not strictly analogous to that for a weak acid but the entropy values −21.7 ± 2.1 eu and −27 2 ± 1 8 eu respectively are in the proper range for such ionizations The entropy of ionization of magnesium ion from biologically important phosphates is surprisingly close to the value for weak acids. The value for magnesium glucose-1-phosphate is −21.1 eu (*99*) and that for magnesium glycerol-2-phosphate is −22 9 eu (*99*)

The reaction between oppositely charged ions is the reverse reac-

tion of ionization and so the entropies have large positive values *(100)*

When an ion reacts with a neutral molecule to produce another ion or when charges are not neutralized or produced, the entropy change may be either positive or negative but is usually small, though not always *(101)*. For example, hydrogen ion reacts with methylamine with an entropy change of 4 7 eu; iodide ion reacts with iodine with entropy change of −4 eu *(101)*.

No charges are neutralized or produced when pyruvate ion is reduced by DPNH so the entropy, 16 5 eu *(102)*, is larger than might be expected at first. An important factor must be the more negative entropy of solvation of the small hydrogen ion than of DPN^+. [Cf. Eq (10) for relation of solvation entropy to radius of ion.]

F. Entropy of Activation during Reaction

The entropy of activation *(103)* is the entropy difference between the entropy of the transition state and the entropy of the reactants. The transition state occurs during the reaction when the energy is the highest and the molecules are about halfway between reactants and products. The entropy of activation affects the rate of the reaction because it contributes to the free energy of activation (ΔF^*); and the rate constant, k, is proportional to $e^{-\Delta F^*/RT}$

The entropy of activation is determined by measurements of the rate of reaction at a series of temperatures The entropy of activation depends on the heat and free energy of activation

$$\Delta F^* = \Delta H^* - T\Delta S^* \qquad (12)$$

Therefore, ΔS^* may be calculated from ΔF^* obtained from a value of the rate constant at one temperature

$$\Delta F^* = RT \ln k + RT \ln \left(\frac{kT}{h}\right) \qquad (13)$$

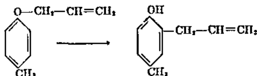
and ΔH^* obtained from the dependence of ΔF^* on temperature.

$$\Delta H^* = RT^2 d \frac{\ln k}{dT} - RT \qquad (14)$$

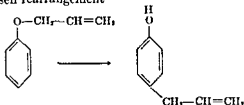
In the above equations T is the absolute temperature, R is the gas constant, K is the Boltzman constant, and h is Planck's constant. Note the resemblances between Eqs. (12), (13), and (14) and Eqs. (2), (5) and (7), respectively.

Entropies of activation often give clues as to reaction mechanisms

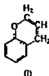
Both the o-Claisen rearrangement

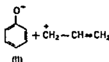


and the p-Claisen rearrangement



have negative entropies of activation The o-Claisen rearrangement could proceed through a cyclic intermediate (I) or by a dissociation into ions II.





The negative entropy of activation, −8 1 eu (*104*) supports the first mechanism which should produce a decrease in entropy in going to the transition state, rather than the second one which should produce an increase The p-Claisen rearrangement also has a negative entropy of activation, −10 1 eu (*104*), in agreement with a cyclic transition state. Subsequent work has shown that the rearrangement first forms the o-derivative and then the para so that the initial steps in the rearrangements are identical.

The entropies of activation of unimolecular ionizations



$$AB \rightarrow A^+ + B^-$$

are again largely dependent on solvation of A^+ and B^- because in the transition state AB is becoming like A^+ and B^-. Large groups on A^+ and B^- decrease the solvation in the transition state and increase the entropy of activation (*105*). In the reverse reaction the arguments are the opposite.

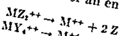
The entropies of activation in [nucleophilic] substitution reactions



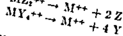
$$X + RY \rightarrow XR + :Y$$

depends upon the substituents on R which affect the solvation o transition state (*105*). Substituents on the carbon atom of the R g from which Y is displaced prevent solvent from solvating the pa positive charge on the R group in the transition state and increase entropy of activation For example, in the reaction of RCl with iod ion in acetone the entropy of activation is -15.0 eu for *n*-propyl chlori and -9.4 eu for sec-propyl chloride (*105*).

Because so many enzymes contain chelated metals, this rathe specialized subject deserves some discussion. Metal chelates of di [or polyfunctional] ligands, Z, are more stable than metal complexes of mono functional ligands, Y, because of an entropy factor.



$$MZ_2^{++} \rightarrow M^{++} + 2\,Z \qquad (I)$$



$$MY_4^{++} \rightarrow M^{++} + 4\,Y \qquad (II)$$

Reaction (II) has a greater increase in translational and rotational degrees of freedom than (I) so that the entropy increase is greater for (II). Other factors than translation also make contributions (*106*) The entropy of dissociation of Zn $(NH_3)_4^{++}$, 2 7 eu (*107*), is much more positive than the corresponding value of -10.7 eu (*107*) for Zn $(en)_2^{++}$ [en = ethylene-diamine] Similarly the value 19 8 eu (*107*) for Cu $(NH_3)_4^{++}$ is mor positive than 0 7 eu for Cu $(en)_2^{++}$ This entropy effect, important whe a molecule is bound at more than one point, must contribute to the enzymatic binding of many substrates and coenzymes

G Entropy Changes during Protein Denaturations

Native protein chains occur in compact configurations such as the α-helix. Upon denaturation these chains unwind or unfold to form long flexible chains The rotational degrees of freedom of the chain are greatly increased and the entropy change during denaturation is a large positive value (*108–110*). The proteins are partially unfolded in the transition state on the pathway to denaturation so that the entropies of activation for protein denaturation are also large positive numbers Luciferase increases in entropy by 196 eu (*111*) on denaturation The entropy of activation is 118 eu (*111*) The entropy of activation for thermal denaturation of hemoglobin varies from 152 7 eu to 319 5 eu depending upo pH and solvent It is also large for the thermal inactivation of viruse varying from 100 to 250 eu for the bacteriophages (*112*).

Freeze denaturation of proteins and thermal denaturation in the dry state give negative changes of entropy during reaction or activation For example, the reversible inactivation of the enzymes peroxidase and phosphatase at low temperatures occur with large decreases in entropy (*113*). The values ranges from -65 eu to -100 eu depending upon pH.

These values are interpreted as inactivation accompanied by an increase in hydrogen bonding. In the dry state the thermal inactivation of deoxyribonuclease (*114*) was found to have an entropy of activation equal to −37 eu The thermal inactivation of bacterial viruses in the dry state also has a negative entropy of activation The mechanism of protein denaturation must be very different with dry than with wet proteins

H. Entropy Change during Enzyme Reactions

Studies of the entropy of enzyme-substrate complex formation gives some information as to the size of the "reaction segment" (*111, 115*) i e, how much of the protein structure is reorganized during the enzymic catalyses Does a portion of the protein reorganize to fit the substrate or does the enzyme act as an inflexible template?

A study of the trypsin-soybean trypsin inhibitor reaction is instructive Soy bean trypsin inhibitor may be considered a substrate with a very low rate of hydrolysis The entropy increase (*116*) [15–20 eu] may be explained by either a freeing of water of hydration or a reorganization of the enzyme to fit the substrate McLaren (*117*) has measured the actual water released dilatometrically during this reaction and has found that the increase in water volume cannot explain all the entropy increase found.

The enzymes urease and pepsin both form complexes with positive entropies (*118, 119*) For example, the entropy of formation of the pepsin-carbobenzoxyl-l-glutamyl-l-tyrosine ethyl ester complex is 20 6 eu and that of the urease-urea complex is 13 3 eu However, this latter value falls to 0 4 eu in a different buffer at a different pH (*120*). The positive entropy of formation has been attributed either to the desorption of water of solvation of the enzyme or to a slight disorganization of the protein structure to fit the substrate, i e, the enzyme has an appreciable reaction segment Casey and Laidler prefer this latter explanation The entropies of activation for the reactions of the complex to give a product is slightly negative This is explained by a reorganization of the enzyme during this process The entropy of the formation of various inhibitor-chymotrypsin complexes have been found to be fairly small and for most inhibitors negative (*121*) The formation of these enzyme-inhibitor complexes could have a small reaction segment

The ΔS values of hemoprotein reactions reflect changes in hydration and configuration (*98*) In myoglobin the protein participates by tightening its structure as the water molecule coordinated to the Fe is replaced by another ligand such as O_2.

Glucose dehydrogenase combines with the inhibitor urethane, with a small positive entropy, 2 75 eu The ΔH is zero so that the entropy is the sole contributor to the ΔF of the reaction (*122*).

The entropies of complex formation between alcohol dehydrogenase and substrates are small but vary in sign depending upon the substrate and the pH (*123*).

Interpretation of all the entropies of enzyme-substrate complex formation are unclear because of the water of hydration released on formation of the complex. A quantitative treatment of the hydration problem would be an important contribution to enzymology.

An important function of enzymes may be to hold reactants in the proper position for reaction so that the entropy of activation does not contain the large negative term due to loss of freedom of reactants going into the activated state; thereby making ΔS^* more positive during the actual reaction on the enzyme surface. For example, in the pepsin-catalyzed hydrolysis of a glutamyl tyrosine peptide the enzyme may increase the reaction velocity only by increasing the entropy of activation. The pepsin-catalyzed hydrolysis has a heat of activation (*119*) of 23.1 ± 1.2 *K* cal which is quite comparable to that found for the acid catalyzed hydrolysis of peptides, 19.7 *K* cal. However, the entropy of activation (*124*) (starting with free enzyme and free substrate) is 16.1 eu for the enzyme and −16.6 eu to −27.1 eu for acid-catalyzed peptide hydrolysis. A similar thermodynamic relationship explains the greater efficiency of ion exchange resins over acid in catalyzing the hydrolysis of peptides (*125*).

Mechanisms have been proposed with an attempt to provide for a more positive entropy of activation for several enzyme-catalyzed reactions, In general these mechanisms allow for the unfavorable entropy change to occur during the enzyme-substrate complex formation. The rate-determining step occurs after the substrates are absorbed on the enzyme so that there is a more positive entropy of activation. Mechanisms of enzymic reactions with favorable entropies of activation have been proposed for hydrolytic reactions (*126*), enzymic reactions involving adenosine triphosphate (*127*), and the condensation of acetyl coenzyme A with oxalacetic acid (*128*).

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The Mitochondrial System of Enzymes

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I. Biochemical Machines

The transformation of energy from one form to another is accomplished in living systems by specialized structures which we may refer to as biochemical machines. The chloroplast system of green plants converts radiant energy into the chemical energy of ATP, the mitochondrion couples the synthesis of ATP to the oxidation of pyruvate; structures in the eye and ear convert light and sound energy into the electrical energy of nerve excitation; structures in the cell membrane and kidney couple the selective movement and concentration of ions and solutes to oxidative processes, the neural apparatus initiates electrical excitations by appropriate chemical reactions, while in muscle an electrical stimulus triggers chemical processes which lead to mechanical shortening All these energy transformations are brought about by intracellular structures of a high degree of complexity The understanding of how these machines function requires the knowledge of how they are constructed and how the component parts are put together.

Enzymic reactions underlie all biological energy transformations, but they lose much of their meaning when considered out of the context of the structures in which they proceed For example, the ATPase reaction of the contractile protein is in an over-all sense indistinguishable from an analogous process catalyzed by a large group of soluble enzymes. But the intimate association of ATPase with the contractile protein of muscle imparts a new meaning and significance to this enzyme which it does not have when considered purely in the context of an enzyme *qua* enzyme ATPase activity of myosin is intimately tied up with the mechanism of muscular contraction (*1*), and the molecular changes in myosin or associated macromolecules which accompany ATPase activity are elements just as essential in the catalysis as the enzymic process itself Thus the structure of the biochemical machine not only facilitates the enzymic process which leads to the energy transformation but also provides for the molecular contiguity of the energy-transforming system and the system which accepts or is activated by the energy so transformed The study of biochemical machines thus clearly lies at the crossroads of physiology, biochemistry, and physics

A. The Function of the Mitochondrion

The cells of all aerobic organisms, animal, plant, and microbial, contain a rod- or spherical-shaped body called the mitochondrion which

provides in a utilizable form the energy required for basic cellular functions. Energy derived from the oxidation of pyruvate to CO_2 and water by way of the citric acid cycle is stored as it were in the form of the bond energy of ATP. The mitochondrion is thus primarily a device for coupling the synthesis of ATP to the five oxidative reactions of the citric acid cycle (*2–4*) The universal attribute of all mitochondria is this coupling capacity which is referred to as oxidative phosphorylation But oxidative reactions other than these of the citric cycle may also be coupled Thus, for example, in the mitochondria of the thoracic muscle of the housefly the oxidation of α-glycerophosphate to dihydroxyacetone phosphate is quantitatively more important in oxidative phosphorylation than citric cycle oxidations (*5*). In heart mitochondria the oxidation of β-hydroxybutyrate to acetoacetate is a coupling reaction additional to those of the citric acid cycle In different microorganisms one or another of a large variety of substrates seem to be capable of coupled oxidation, and it would thus appear that the constant in mitochondria is the coupling phenomenon while the variable is the nature of the oxidative reactions which are coupled All mitochondria without any known exception catalyze the citric acid cycle, but this is not necessarily the predominant or exclusive coupling process

In addition to the basic mechanism for oxidative phosphorylation the mitochondrion may contain systems which catalyze ATP-dependent syntheses or conversions For example, liver mitochondria contain the system for condensation of benzoyl CoA and glycine to form hippuric acid (*6, 7*). This synthesis requires ATP for the conversion of benzoate to benzoyl CoA Animal mitochondria generally contain a system which catalyzes the synthesis of lecithin or cephalin by the condensation of cytidine diphosphocholine or ethanolamine with α,β-diglycerides (*8*) ATP is required for the generation of both partners in the condensation. The system for the complete oxidation of fatty acids is localized exclusively in the mitochondria of animal tissues (*2, 3*) ATP is required for the conversion of the fatty acid to its acyl CoA ester (*9*), and the two-carbon unit formed by β-oxidation (acetyl CoA) is in fact one of the intermediates in the citric acid cycle Thus the structural association of the fatty acid oxidizing system with the citric acid oxidizing system of the mitochondrion underlies the functional interrelationships existing between these two systems

B The Mitochondrion as a Complete Biochemical Unit

The mitochondrion contains the complete set of enzymes, cofactors, and accessory substances required to implement all the primary and ancillary reactions which it catalyzes (*2–4*) When prepared with proper

precautions the mitochondrion requires the addition only of the appropriate substrates, inorganic phosphate, ADP, and molecular oxygen. Everything else is built into the mitochondrion This concept of the mitochondrion as a complete, organized unit was accepted only belatedly by biochemists, but the supporting evidence is now so overwhelming that the issue can be considered to have been finally resolved (*10a*).

The basic mitochondrial functions (citric cycle oxidations, electron transport, and oxidative phosphorylation) require the collaboration of at least twenty different enzymes, and allowing for auxiliary functions the total in some mitochondria may easily come up to forty enzymes. The most reasonable interpretation of the available data is that all the enzymes in the mitochondrion are arranged in a unique pattern, and that there are colligative forces ranging in strength from van der Waals' forces to covalent bonds which keep the enzymes constrained within that pattern. There are, in addition, structural devices analogous to clathrate formations which also contribute to maintaining the integrity of the mitochondrial pattern of enzymes.

The arrangement and organization of enzymes within the mitochondrion should not be looked upon merely as a device to speed up chemical reactions Nature has found much more effective and far less cumbersome ways of speeding up an enzymic process The structural pattern has to be interpreted in terms of the function of the mitochondrial machine, namely oxidative phosphorylation. That is to say, the arrangement is designed to implement this function and indeed it is only in terms of arrangement that the mechanism of function can be deduced.

C. Mitochondrial Form and Size

The mitochondrion is a cigar-shaped body with a system of external and internal double membrane structures There are two ways of interpreting the structure of the mitochondrion According to Fig. 1a the mitochondrion consists of an external limiting membrane (with double membrane structure) enclosing an array of closed vesicles or bags stacked atop one another like saucers. The walls of these vesicles also have double membrane character According to this interpretation the cristae are separate from the external membrane. Figure 1b is an alternative interpretation of mitochondrial structure. According to this version the cristae are extensions of the inner wall of the external double membrane.

If there are separate cristae then compartmentalized within each crista would be intracristael fluid whereas if the cristae are extensions of the inner wall of the external membrane the intracristael fluid would be continuous. In both cases, however, the intercristael fluid is contin-

uous At the present time it is difficult to decide between these two interpretations.

Perhaps the important point is that the structural elements within the mitochondrion are in contact with a fluid milieu which is sealed off from the external medium and which contains essential enzymes and cofactors When the mitochondrion is structurally damaged the enzymes and cofactors present in the interior leak out and there is a corresponding loss of enzymic function.

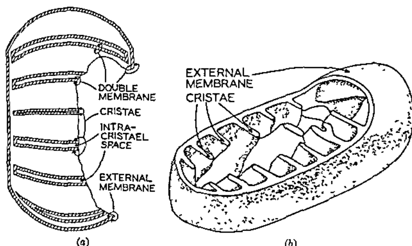


FIG 1 Two diagrammatic representations of the mitochondrion The model in Fig 1b is taken from Palade (*10b*)

The long dimension of mitochondria may vary from 0 5 to 3 μ while the short dimension may vary from 0 1 to 0 5 μ. The precise size of the mitochondrion may be an accident like the size of a starch granule. Motion picture studies of living fibroblasts show that two or more mitochondria can coalesce to form a giant mitochondrion, or any one can undergo fragmentation to two or more daughter particles (*15*) These cinematographic studies suggest· (1) that the mitochondrion is built up of a material of great plasticity; (2) that the double-membrane system of one mitochondrion can coalesce with the system of another; (3) that after coalescence, external double-membrane structures become cristae while during fragmentation the reverse takes place, and finally (4) that the structural unit of the mitochondrion is clearly not the smallest functional unit or the least common denominator of mitochondrial function

Some mitochondria, e g , those of heart and the flight muscle of the insect, are jam-packed with cristae (*16, 17*) which are arranged like logs

on a pile Others, like those of liver, have relatively few cristae, and there are wide spaces between successive cristae (*11*). In general it appears that the greater the number of ancillary reactions catalyzed by the mitochondrion the smaller the number of cristae per mitochondrion Mitochondria, however, which do no more than the basic coupling reaction of oxidative phosphorylation usually show a tight arrangement of packed cristae Naturally these are more active per milligram protein in catalyzing citric cycle oxidation or oxidative phosphorylation than mitochondria with additional enzymic functions.

The status of mitochondria in bacteria has been an uncertain one for some years, but now that the mitochondrion is being looked upon as a functional unit independent of size or shape, the difficulty may be resolved From the standpoint of mitochondrial function there can be no doubt that bacteria contain particulate entities which correspond in every respect to classic mitochondria (*18, 19*) As was discussed above, the size of the particle is immaterial as far as function is concerned over wide limits

There is great variation among mitochondria from different sources with respect to the deformation which can be tolerated without loss of activity Freezing and thawing of liver mitochondria leads to rapid loss of phosphorylating activity (*20*), but the same treatment has relatively little effect on heart mitochondria (*21*).[1] Heart mitochondria can sustain considerable osmotic deformation without significant loss of phosphorylative activity Two broad generalizations based on studies of heart mitochondria are pertinent to this discussion: (1) the capacity to carry out the complete citric cycle is lost when mitochondrial form is obliterated (*16*), and (2) the capacity for oxidative phosphorylation disappears when double-membrane structure is lost (*16*). Thus mitochondria which have exploded or have undergone fragmentation to particles without the characteristic arrangement of limiting membrane and cristae can no longer carry out the complete citric acid cycle, but they are capable of coupling the oxidation of DPNH or succinate to phosphorylation providing the fragments still have a double-membrane structure Once this is gone, electron flow is uncoupled from phosphorylation even though the oxidative functions may be unaffected

D UNIT OF MITOCHONDRIAL ACTION

Heart mitochondria can be comminuted to smaller fragments which can best be described as miniature mitochondria (*23*). These retain the essential features of mitochondrial form such as an external membrane

[1] Privitera *et al* (*22*) have found that slow freezing and storage of liver mitochondria at very low temperatures can preserve oxidative phosphorylation

and internal cristae (*16*). The enzymic properties of these particles are qualitatively indistinguishable from those of intact, unmodified mitochondria When the comminution of mitochondria is carried out under somewhat different conditions, much smaller fragments are formed that no longer retain any vestige of mitochondrial form but still show double-membrane structure (*24*) The electron microscope photographs of these particles with double-membrane structure suggest that these are fragments of the mitochondrial structure (*25*) Since the conversion of mitochondria to these particles can be almost quantitative it is reasonable to assume that they are derived from the double-membrane structures of both external membrane and cristae Still under other conditions mitochondrial fragments can be obtained which no longer show either mitochondrial form or double-membrane structure These appear as single-layered vesicles in electron microscope photographs without any evidence of internal structure (*16*) The third of this trio of particles is called the electron transport particle or ETP (*16*, *26*), and the second with phosphorylating properties and double-membrane structure is referred to as ETP_H (*16*, *24*), the subscript "H" serving to distinguish the phosphorylating from the nonphosphorylating form of ETP.

The electron transport particle or ETP may be looked upon as a mitochondrial subunit from which most of the enzymes concerned in citric cycle oxidations and oxidative phosphorylation have been stripped. It still retains a complete electron transfer chain and catalyzes the oxidation of succinate or DPNH by molecular oxygen ETP_H is a subunit which like ETP has lost all but one of the enzymes for citric cycle oxidation, but unlike ETP it can couple the oxidation of succinate or DPNH to esterification of inorganic phosphate and synthesis of ATP Thus the enzymes concerned in oxidative phosphorylation are still functional in the particle

The results of the studies described above and of other similar studies may be interpreted in the following way The mitochondrion is a polymer of a repeating unit which contains each of the component enzymes and coenzymes in some simple stoichiometric molecular relation If, for the sake of simplicity, we assume all components in a 1 1 molecular ratio then the unit would contain one molecule of each enzyme in the mitochondrion In a mitochondrion of average size there would be several hundreds, if not thousands, of such units The degradation of a mitochondrion to this unit is probably impossible of attainment since the forces required to separate one unit from its neighbor are strong enough to detach enzymes from a single unit But it is possible to degrade the mitochondrion to a subunit like ETP which contains only the tightly associated enzymes of the electron transport system The rest of the

enzymes are jettisoned during the fragmentation procedure. In principle, particles of ETP should be capable of subdivision until they attain the dimensions of a single unit since there is no reason known why the activity of a single such unit should not be the same as that of a polymer. But this is clearly not the case for ETP_H. The requirement of a double-membrane structure imposes a lower limit below which phosphorylating activity cannot be retained, and this lower limit is probably well above the molecular dimensions of a single unit. Thus in this case the functional unit and the molecular unit do not coincide, and the greater the complexities of the functional unit the greater the divergence.

II. Electron Microscope Studies

The application of electron microscopy to the study of the intimate structure of mitochondria was pioneered by Palade (*11, 12*), Sjöstrand (*13, 14*), and Dalton (*27*), and this tool has greatly facilitated the interpretation of the sequence of events which takes place during the fragmentation of mitochondria. The material to be examined is "fixed" with osmium tetroxide, embedded in methacrylate and then sectioned with a glass edge The preliminary preparation as well as the conditions for electron microscopy are frankly brutal, and the possibility of artifactual structures being introduced is one that cannot be ignored.

A set of nine electron micrographs is shown in Figs. 2 to 8 and 10 and 11. In the intact heart muscle the mitochondria are lined up between muscle fibers like rows of soldiers The double-membrane structure of the limiting membrane and cristae are clearly seen (cf. Fig. 2). When mitochondria are torn out of the muscle fibers, during homogenization of the heart muscle in a mechanical blendor, they retain their size and arrangement only when exposed to suitable media such as 0 88 *M* sucrose (cf. Fig 3). In 0.25 *M* sucrose they tend to swell up into bloated forms; the external membrane pulls away from the cristae, and the arrangement of cristae becomes irregular (cf. Fig. 4). Mitochondrial suspensions in 0 25 *M* sucrose can be separated into two fractions—the light fraction which consists predominantly of bloated mitochondria in which the cristae are massed in one or two pockets (cf. Fig. 5) and the heavy fraction which is jam-packed with cristae no longer arranged in an orderly manner (cf. Fig 6). The light mitochondrial fraction can be further fragmented into two major particle types—one of which corresponds to a miniature mitochondrion (cf. Fig. 7) while the other is the electron transport particle (cf. Fig 8). The miniature mitochondrial particle was originally called the phosphorylating electron transport particle or PETP, but since this name may lead to confusion with ETP in a phosphorylating



FIG. 2 Electron micrograph of a section of heart muscle Ph
D. L. Filmer and Paul Kaesberg of the University of Wisc
45,000 diameters

form, we shall refer to the partıcle merely as a mmıature mıtochondrion It has an external lımıtıng membrane and crıstae—both with double-layer structure ETP has a sıngle-membrane vesıcular structure Fıgure 9



FIG 3 Electron mıcrograph of a suspension of heart mıtochondrıa ısolated ın 0 88 *M* sucıose Magnıfication about 60,000 dıameters From Zıegler *et al* (*16*)

ıs a dıagrammatıc representatıon of the stages ın the conversıon of a swollen mıtochondrıon to the varıous derıvative partıcles

When the heavy mıtochondrıal fractıon ıs exposed to sonıc ırradıatıon after freezıng and thawıng, the fragmentatıon process takes the course clearly outlıned ın Fıgs 10 and 11 After freezing and thawıng the mıto-



FIG. 4 Electron micrograph of a suspension of heart mitochondria isolated in 0 25 *M* sucrose Magnification about 73,000 diameters Photograph prepared by D L Filmer and Paul Kaesberg of the University of Wisconsin



FIG 5 Electron micrograph of a section through the light mitochondrial fraction Magnification about 60,000 diameters From Ziegler *et al* (*16*)

chondrion becomes distorted, the external membrane may become torn, and some of the cristae have been jettisoned (Fig 10) After irradiation the mitochondria disintegrate, and only fragments of double-membrane



FIG 6 Electron micrograph of a section through the heavy mitochondrial fraction Magnification 50,000 diameters From Ziegler *et al* (*16*)

structures survive (Fig 11) These are the phosphorylating particles which have been designated as ETP_H

III. The Concept of Supramolecular Particles

The mitochondrion can be visualized, and indeed it behaves as a giant macromolecule or supramolecule which is no different except in



FIG 7 Electron micrograph of a section through a suspension of miniature mitochondria Photograph taken by H Ris and C M S Dass of the University of Wisconsin

respect to size from macromolecules such as proteins It resembles in many respects polymeric macromolecules such as nucleic acid and glycogen, though admittedly the complexity is of a higher order of magnitude The mitochondrion can be fragmented to smaller particles—still



Fig 8 Electron micrograph of a section through a suspension of ETP Magnification 48,000 diameters From Ziegler *et al* (*16*)

polymers—which are qualitatively indistinguishable from the parent particle in respect to chemical composition and enzymic activity But

parent particle. This process of subdivision and comminution can be extended further Not only can the polymer be subdivided mechanically until the limiting monomer stage is approached, but the monomer which is a complex of twenty or more enzymes can be cleaved in some regular fashion We may express the two different ways of degrading the mitochondrion by Diagrams 1 and 2 in which the dotted lines indicate the

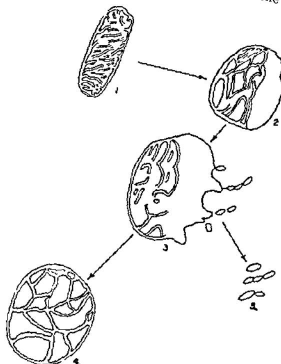


FIG 9 Schematic representation of the sequence of events which lead to the formation of the various particles present in the mitochondrial suspensions prepared in 0 25 *M* sucrose 1 Heart mitochondria as they appear in normal heart muscle 2 Mitochondria within the muscle after exposure to 0 25 *M* sucrose The outer membrane is pushed away from the cristae, and there is progressive swelling of the intercristael areas 3, 4, and 5 These three forms are present in heart mitochondria isolated in 0 25 *M* sucrose Number 3 is a reproduction of a crescent mitochondrial form showing the vesiculation of the outer membrane The transition from 2 to 3 occurs when the mitochondria are stripped from between-the-muscle fibrils It is during this process that portions of the outer membrane are pinched off to form the vesicles characteristic of the ETP fraction (*5*) If all or most of the ballooned part of the outer membrane is stripped off, the cristae encased in the remaining portion of the outer membrane give rise to the characteristic particle of the heavy mitochondrial fraction (*4*)



FIG 10 Electron micrograph of a suspension of heart mitochondria in 0 25 *M* sucrose after freezing and thawing Photograph taken by D L Filmer and Paul Kaesberg, University of Wisconsin

The dehydrogenase catalyzes both the oxidation of succinate by the acceptors enumerated above and also the reduction of fumarate by leuco-dyes such as leucodiethyl safranin (*32b*).

C DPNH Dehydrogenase

This enzyme has been isolated in three different forms: as (a) cytochrome *c* reductase (*33–35*), (b) Straub diaphorase (*36, 37*), and (*c*) the lipoflavoprotein of Ziegler *et al* (*38*) The available evidence suggests that forms (a) and (b) are derived from the parent lipoflavoprotein[2] When the lipoflavoprotein is treated with aqueous butanol and salt it can be stripped of lipid and converted to a flavoprotein which closely resembles the Straub diaphorase The derivative flavoprotein has a molecular weight of about 70,000, assuming one mole of flavin per mole of protein (*39*), and we shall designate it as flavoprotein$_Z$ (Z for Ziegler) to avoid confusion There are four atoms of iron associated with each mole of flavoprotein$_Z$ The Straub diaphorase like flavoprotein$_Z$ catalyzes the oxidation of DPNH (but not TPNH) by various electron acceptors such as methylene blue and indophenol Cytochrome *c* is inactive as electron acceptor for the dehydrogenase system. The flavin prosthetic group which is detached from the protein under acid conditions has been identified as flavin adenine dinucleotide (*37*)

When the flavolipoprotein or at least its counterpart in the particle is exposed to pH 4 8 in presence of 10% alcohol at 38° a flavoprotein is detached in the form of a cytochrome *c* reductase which catalyzes the oxidation of DPNH by cytochrome *c* as efficiently as by ferricyanide (*34, 35, 38*) In the course of this treatment the prosthetic group of the flavoprotein is modified chemically and though still a dinucleotide of flavin and adenine no longer shows activity in assay systems which are specific for flavin adenine dinucleotide (*39*)

The flavolipoprotein is about ten times more active than the cytochrome *c* reductase in the DPNH-ferricyanide assay system (low levels of acceptor) and several hundred times more active than the Straub or Ziegler diaphorase (*38, 39*) Apparently the lipoflavoprotein is the only one of the three forms of the enzyme which is capable of interacting at

[2] According to a recent study of V Massey (*38a,b*) there are two separate flavoproteins one of which is identical with the Straub diaphorase and has the property of catalyzing the oxidation of DPNH by thioctic acid or derivatives thereof while the other, which shows no such activity, may be identified with the lipoflavoprotein and cytochrome *c* reductase The thioctic reductase is presumed to work physiologically in the reverse direction. That is to say it catalyzes the reduction of DPN by reduced thioctic acid By contrast the other flavoprotein catalyzes the oxidation of DPNH by some oxidation-reduction component in the electron transport chain

maximal velocity with ferricyanide at low levels of acceptor With methylene blue as acceptor all three forms of the dehydrogenase have comparable activities per mole of flavin

The prosthetic group of DPNH dehydrogenase is split off quantitatively from the apoenzyme under acid conditions (5% trichloroacetic acid) (*35*) This property sharply distinguishes between the respective prosthetic groups of succinic and DPNH dehydrogenase Differential extractability has provided the basis for determining the amounts of the two dehydrogenases in a given particle preparation

The lipoflavoprotein form of DPNH dehydrogenase has a molecular weight which is a multiple (at least four times) of the molecular weight of the smallest common denominator (the dry weight which contains one mole of flavin) (*40*) This discrepancy in molecular weights has been found to apply to all the other components of the electron transfer sequence, and it is the foundation stone for the hypothesis of the polymolecular structure of the ultimate units of these components (*10a*) According to this hypothesis the unit of DPNH dehydrogenase contains at least four moles of the flavoprotein which are complexed to a lipoprotein The properties of this polymolecular unit may be quite different from those of the "unimolecular," classical form of the flavoprotein

D THE CYTOCHROMES

The cytochromes are a group of hemoproteins discovered by Keilin in his classic researches on the pigments of aerobic organisms (*41–48*) Cytochromes *a*, *b*, and *c* were the first three recognized and characterized by Keilin In 1941 Okunuki and Yakushiji discovered the fourth member of the group which is now referred to as cytochrome c_1 (*49, 50*) The structural formulas of the heme groups (*51a*) of cytochromes *a* (*51b, 52a*), *b* (*52b*), and *c* (*53–55*) are shown in Fig 13 The prosthetic hemes of cytochromes *b* and *a* can be detached from the protein in acid acetone, (*57–59*) but the hemes of cytochromes *c* and c_1 are so firmly bound to their respective proteins that rather drastic procedures are necessary to break the bonds (*56, 59*) The protoporphyrin of cytochrome *c* is probably linked to the protein by means of two thio-ether bonds (*51a, 53, 54*) Cysteine residues in the protein are concerned in these bonds (*60*)

All the four cytochromes have now been isolated in a soluble and homogeneous state The molecular weight of cytochrome *c* is 13,000 (*61*) and it is by far the smallest molecular-weight unit of the four cytochromes Cytochrome c_1 can be isolated as a lipid-free, soluble protein with a molecular weight of approximately 400,000 (*56*) According to Criddle and Bock (*61a*) cytochrome c_1 occurs as a polymer which can be

depolymerized to a soluble monomer of molecular weight 70,000. There are probably 6 molecules of monomeric cytochrome c_1 in the polymer Cytochrome *a* has been obtained in highly purified state by various investigators (*62–65*), but as isolated the hemoprotein is a water-insoluble

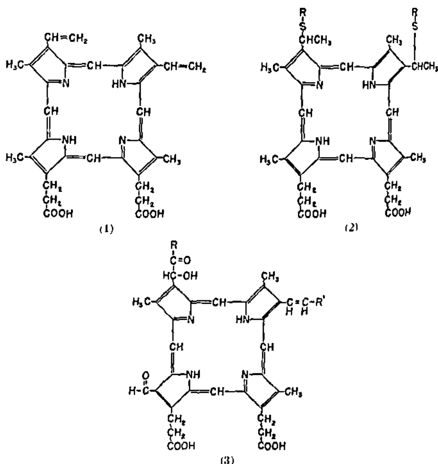


Fig 13 Structural formulas of the prosthetic group of: 1 Cytochrome *b*—Protoporphyrin IX (*52b*) 2 Cytochrome *c*—Substituted mesoporphyrin (*53–55*) The prosthetic group of cytochrome c_1 is probably very similar, if not identical, with that of cytochrome *c* (*56*) 3 Cytochrome *a*—Cytodeuterophorphyrin (*51b, 52a, 52c*)

complex which can be solubilized by deoxycholate and other bile salts or detergents Ambe and Venkataraman (*61b*) have recently succeeded in depolymerizing cytochrome *a* to a water soluble monomer of molecular weight 72,000. Because of the insolubility of the polymer it is not possible to determine how many molecules of the monomer make up a unit of the polymer. The monomeric form of cytochrome *a* contains two molecules

of copper and one molecule of heme per 72,000 grams (*61b, 61c*). Cytochrome *b* has been isolated from beef heart mitochondria in homogeneous state by Bomstein, Goldberger, and Tisdale (*66*) It forms a water soluble adduct with the cationic detergent cetyldimethylethylammonium

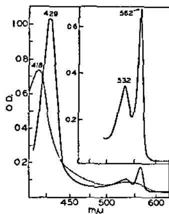


Fig. 14 Absorption spectrum of cytochrome *b* Dashed line, oxidized form, solid line, reduced ($Na_2S_2O_4$) From Bomstein *et al* (*66*)

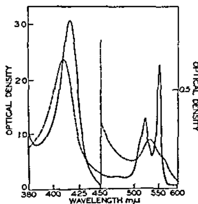


Fig 15 Absorption spectrum of cytochrome *c* Spectra of horse heart cytochrome *c* (Sigma Biochemical Co) Dashed line, oxidized form, solid line, reduced ($Na_2S_2O_4$)

bromide The molecular weight of this derivative of cytochrome *b* is about 20,000. The detergent apparently depolymerizes the polymeric form to the monomeric form which however is still complexed with the detergent. When the heme group of the water insoluble, polymeric cytochrome *b* is removed by acid extraction of the aqueous suspension with methyl

ethyl ketone the heme-free protein becomes water soluble Apparently the water insolubility of the cytochrome is determined in some way by the mode of attachment of the heme group to the protein

The absorption spectra of the four cytochromes are shown in Figs. 14 to 17 Cytochromes c and c_1 have very similar spectra though that of

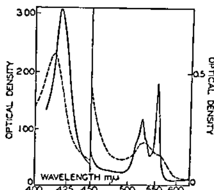


Fig 16 Absorption spectrum of cytochrome c_1. From Green *et al* *(56)* Dotted line, oxidized form, solid line, reduced with dithionite

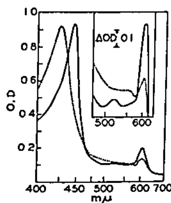


Fig 17 Absorption spectrum of cytochrome a From Ambe *et al* *(61b)* Dotted line, oxidized form, solid line, reduced with dithionite

c_1 is uniformly shifted toward the longer wave lengths At the temperature of liquid air the α-band maximum of cytochrome c is shifted from 550 mμ to 549 mμ *(67, 68)*. Cytochrome c_1 does not show a corresponding shift The spectrum of cytochrome a shows two typical bands—the α at 605 mμ and the Soret at 444 mμ. There is a definite β band at 518 mμ but proportionately much reduced in size compared to the β bands of the other cytochromes *(61b, 61c)*

There is considerable variation in the maxima for the bands of cytochromes in various microorganisms, plants, and animal tissues From a functional standpoint this variation may not be too disturbing The various cytochromes play entirely different and specific roles in the electron transfer process Thus cytochrome a is always concerned in the terminal reaction with molecular oxygen Cytochrome c_1 is interposed between the flavoprotein and cytochrome a whereas cytochrome c is engaged in a shuttle role between c_1 and a It may prove easier to match cytochromes from different tissues in terms of function than in terms of spectra The analogy of the hemoglobins is very much to the point

According to Keilin (*45*), cytochrome a is a mixture of two cytochromes, a and a_3, which overlap closely in spectrum Only a_3 is autoxidizable and can react with cyanide and CO There has been no direct evidence at all for the existence of two separate cytochromes in preparations of cytochrome oxidase, and according to Wainio (*69*) the spectral anomalies pointed out by Keilin may find their explanation in terms other than the existence of separate cytochromes Okunuki *et al* (*70a*), for example, have postulated an oxygenated form of oxidized cytochrome a which reacts with reduced cytochrome c to form reduced cytochrome a and oxidized cytochrome c Okunuki equates the oxygenated form of cytochrome a with cytochrome a_3 In the present review we shall assume only one form for cytochrome a, and the spectral anomalies will be attributed to factors other than a mixture of cytochromes

The brilliant spectroscopic studies of Chance and his group (*70b*) have provided a broad experimental foundation for the detailed analysis of the electron transport system Chance has developed instrumentation for observing the rates of reduction of the cytochromes in both intact cells and in particle suspensions and has perfected the technique of quantitative, rapid flow spectrophotometry in the study of the electron transport process

E Metal Components

At least three metals play a role in the electron transport system of mitochondria These are iron (*26*, *32*), copper (*20*), and magnesium (*71*) Iron is chelated with the porphyrin nucleus, but in addition some 5 to 6 times as much nonheme iron is bound to various components of the electron transfer chain (*26*) Iron is found to be associated with several of the proteins isolated in homogeneous state from the electron transport system such as the succinic dehydrogenase (*29*, *30*) (2 to 4 atoms of iron per mole of protein) and the DPNH dehydrogenase (2 to 4 atoms of iron per mole of protein) (*39*) Beinert and Sands (*71a*, *71b*) have shown by electron spin resonance measurements that in a particle with DPNH-

and though more efficient than cholate or deoxycholate for displacing lipid they are less useful when enzymically active particles are the objective

The detached lipid most often assumes a water soluble form. The state of the lipid is brought about by orientation of the polar lipid molecules with lyophobic groups directed to the interior of the micelle and the

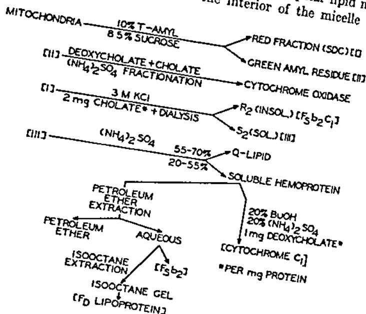


FIG 18 Flow sheet for conversion of mitochondria to various component subunits and summary of conditions for fragmentation

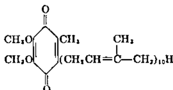
charged polar groups directed to the water phase Some reagents which fragment mitochondria and detach lipid are capable of inducing this oriented state in lipids (*75a*) Thus it is difficult to decide whether the lipid of the mitochondrion preexists in the oriented, water soluble state or is converted to that state in the course of the isolation procedure A lipid system in the water soluble state has the possibility of rapid equilibration with a water phase and it is therefore not unlikely that mitochondrial lipid exists in an ordered state The available evidence suggests that mitochondrial lipid is in very rapid equilibrium with solutes in the water phase

No large differences have been found in the chemical composition of the lipid associated with the different components of the electron transfer chain (*75b*). Ethanolamine and choline phosphatides account for most of

the phospholipid Inositol serine and polyglycerol phosphatides occur in relatively small amounts Almost one of every two molecules of phospholipid contains an acetal linkage (*77*). The high level of plasmalogen may be unique to the mitochondrion of heart muscle The polyunsaturated fatty acids in the lipid of the mitochondrion accounts for a large share of the polyunsaturates found in heart muscle (*78*) The neutral lipid fraction contains a group of as yet unidentified carotenoids, coenzyme Q (a quinonoid component of the electron transport chain), and cholesterol (*77*) The rest of the components of the neutral lipid fraction of mitochondrial lipid have yet to be identified

1 *Coenzyme Q*

Crane *et al* (*79*) discovered in beef heart mitochondria a quinone[1] with the formula shown below



Its structure was determined by the collaborative efforts of the Enzyme Institute and a group from the Merck Sharp and Dohme Research Laboratories (*80, 81*) The compound with a side chain of ten isoprenoid units is referred to as coenzyme Q_{10} Homologs with 9, 8, 7, and 6 isoprenoid units have been isolated from *Torula*, *Azotobacter*, and yeast (*80*). These are referred to as coenzyme Q with the appropriate numerical subscript Plants contain coenzyme Q in their mitochondria (*82, 83*) and an analogous quinone in the green chloroplasts which is structurally related to coenzyme Q but differs in (a) the absence of methoxy-groups in the benzene nucleus and (b) an absorption band for the oxidized form at 254 mμ instead of 275 mμ as in the case of coenzyme Q (*82–84*). The

[1] A form of coenzyme Q was first isolated by Morton *et al* (*86*) as a constituent of [illegible] fat in 1955 [illegible]
[illegible]
a research group of the Hoffman-LaRoche Company in Basel (*88*) It now appears from the studies of Linn *et al* (*89*) from the laboratory of K Folkers that SA was either a mono- or diethoxy-derivative of coenzyme Q_{10} and not Q_{10} itself This explains the difference in the melting point of SA and Q_{10}.

quinone in chloroplasts is a trimethyl benzoquinone analog of coenzyme Q with a side chain of 9 isoprenoid units (*84, 85, 85a*).

Coenzyme Q is reducible by borohydride to its corresponding hydroquinone form, and this in turn can be reoxidized to the quinone by Ag_2O (*90*). The absorption spectra of the oxidized and reduced forms of Q_{10} are shown in Fig. 19. When mitochondria or ETP are extracted with acetone the capacity for oxidizing succinate by molecular oxygen

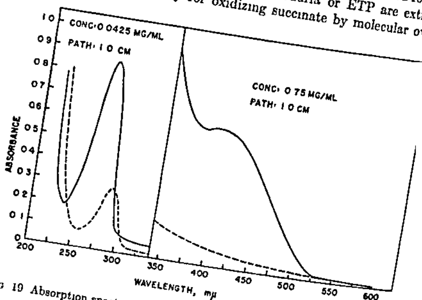


FIG 19 Absorption spectrum of coenzyme Q_{10} From data of Crane *et al* (*79*)

is lost (*91, 92*). This capacity is restored by addition of coenzyme Q (any of its homologs from coenzyme Q_2 to coenzyme Q_{10}). No other con pound yet tested can replace coenzyme Q Cytochrome *c* is required fo this restorative effect, but in absence of Q, cytochrome *c* alone is inactive

It can be shown that during enzymic oxidation of succinate and DPNH in particle preparations coenzyme Q undergoes a cycle of reduction and oxidation (*93*) It is reduced by substrate and in turn the reduced form is oxidized by oxygen This behavior is identical with that of the cytochromes and flavoprotein, and it establishes coenzyme Q as the newest member of the electron transport system Reagents which inhibit the reduction and/or oxidation of the cytochromes also inhibit the reduction and/or oxidation of coenzyme Q, e g, antimycin, cyanide, and Amytal (*93, 94*). The steady state of coenzyme Q like that of mitochondrial DPN is also influenced by the presence of inorganic phosphate and ADP. In presence of phosphate and in absence of ADP, coenzyme Q

accumulates in the reduced form In presence of ADP the oxidized form accumulates (*94*)

2 *Naphthoquinone*

Although coenzyme Q is the only quinone yet recognized in the mitochondria of animal and plant tissues, the status of the quinone in bacterial particles which correspond to mitochondria has yet to be defined satisfactorily Some bacteria like *Azotobacter* contain coenzyme Q_8 and no naphthoquinone (*95*), whereas others like *Mycobacterium phlei* contain a naphthoquinone but no coenzyme Q (*96*) Some bacteria may contain both (*97*) Brodie and his colleagues have implicated a naphthoquinone derivative in *Mycobacterium phlei* as an essential part of the respiratory chain of the organism (*96*) When the quinone is inactivated by ultraviolet light respiration and phosphorylation are abolished Addition of the naphthoquinone isolated from the organism restores both these functions (*98*) The 2-methyl-1,4-naphthoquinone has a side chain with probably 30 or more carbon atoms in the 3-position (*96*) The length of the carbon chain is critical as far as supporting enzymic activity Coenzyme Q does not replace the naphthoquinone in the *Mycobacterium phlei* assay system (*99*), and the reverse is true for the beef heart mitochondrial assay system (*100*).

3 *α-Tocopherol*

Donaldson and Nason (*101*) and Bouman and Slater (*102, 103*) have demonstrated α-tocopherol as a constituent of heart mitochondria present in amounts comparable to those of flavin There is no evidence yet that α-tocopherol undergoes oxidation since it remains in the same form whether the particle is kept anaerobic in presence of substrate or aerobic in presence of oxygen The precise locale of α-tocopherol in the heme chain has yet to be established According to Nason, particles from which α-tocopherol has been extracted under appropriate conditions lose the capacity to catalyze the reduction of cytochrome *c* by succinate and DPNH, and this capacity is restored specifically by addition of α-tocopherol (*104, 105*) However, there are recent reports that these effects of α-tocopherol are artifactual in the sense that they have no relevance to the possible oxidoreduction role of the vitamin in electron transport (*106–108*). Solvent-extracted particles show unusual requirements for activity if the solvent is still present in the preparation. The requirements disappear when the removal of solvent is complete (*106*).

4 *Lipid Cytochrome c*

Cytochrome *c* has been isolated as a water-soluble protein of molecular weight 13,000 (*43, 61*), but in the mitochondrion and derivative par-

ticles, cytochrome *c* exists in the form of a lipid complex (lipid cytochrome *c*) which is insoluble in water and soluble in nonpolar solvents The so-called bound form of cytochrome *c* is in fact the lipid complex (*76*) When particles are treated with phospholipase (*109*) the lipid complex of cytochrome *c* is decomposed with release of water-soluble cytochrome *c* (*110*) Much the same is accomplished by exposing particles containing bound cytochrome *c* to the action of aqueous butanol (10% v/v) Lipid extracted from whole mitochondria takes up relatively large amounts of water soluble cytochrome *c* and converts it to a lipid soluble complex

G Composition of the Electron Transfer Particle (*111, 112*)

The molecular proportions of the known oxidoreduction components of ETP are as follows. For every two moles of flavin there are approximately two moles of cytochromes *b* and *a*, and one mole each of cytochromes *c* and c_1 (*24*) Half the flavin is accounted for as the acid-extractable flavin characteristic of the DPNH dehydrogenase and the rest as the non-acid-extractable form characteristic of succinic dehydrogenase The concentrations and molecular proportions of flavin, heme, coenzyme Q, copper, and nonheme iron are given in Table I

Table I
Composition of ETP[a]

Constituent	Mμmoles or mμatoms/mg protein	Molecular or atomic ratio
Total flavin	0 64	1 0[b]
Cytochrome *a*	0 78	1 2
Cytochrome *b*	0 72	1 1
Cytochrome $c + c_1$	0 60	0 94
Iron (non-heme)	20 3	32
Copper	2 5	3 9
Coenzyme Q	3 1	4 9
Lipid	34 5% dry weight	

[a] Cf references *24, 26 79*
[b] Flavin is arbitrarily taken as 1 0

Since all but one of the protein oxidoreduction components of the electron transfer chain occur as polymolecular units a convention has to be adopted for representing the stoichiometry of these components Let f_s represent the succinic dehydrogenase, f_D the DPNH dehydrogenase and Fe with the subscripts a, b, c_1, and c the corresponding cytochromes The polymolecular unit of f_D will be represented by the symbol $[nf_D]$ where

n is a number probably greater than 4 which represents the number of flavoprotein molecules each containing one flavin group The symbol also expresses the notion that the unit may contain components other than the flavoprotein (e g , lipid and metal) which contribute to the structural character of the unit and which may be indispensable to the functional properties

On the basis of the stoichiometry discussed above the composition of ETP may be formulated as follows:



$$[nf_S][nf_D][nFe_b][nFe_{c_1}](Fe_c)_n[nFe_a]$$

It has yet to be demonstrated directly that the value of n is the same or nearly the same in each unit This would be anticipated in view of the whole number stoichiometry For ease of representation the formulation of ETP can be simplified to the form.



$$(f_S)(f_D)(b)_2(c_1)(c)(a)_2$$

This formula represents the molecular proportions of each of the prosthetic groups Thus c_1 is not one molecule of the cytochrome, but the moiety thereof which contains one mole of heme However, in the case of c the molecule and the unit which contains one mole of prosthetic group are the same

In the mitochondrion the ratio of cytochromes (a $b \cdot c_1 + c$) is the same as for ETP However, the flavin heme ratio is about 1.2 in mitochondria compared to 1 3 in ETP There are two additional flavoproteins associated with the electron transfer chain of beef heart mitochondria viz the fatty acyl CoA dehydrogenase and the electron transfer flavoprotein Both of these enzymes which are concerned with fatty acid oxidation are largely lost in the conversion of mitochondria to ETP according to Beinert and Lee (*113*)

V. Fragmentation of ETP

The mitochondrion or ETP can be fragmented into a series of less complex particles which do not contain all the oxidation-reduction components of the electron transfer chain A partial list of these particles with their approximate composition is given below·

$(f_S)(f_D)(b)_2(c_1)(c)$	succinic-DPNH bc_1 complex (*114*)
$(f_S)(b)_2(c_1)$	succinic bc_1 complex (*74*)
$(f_D)(b)_2(c_1)(a)_2$	green particle (*116*)
$(f_D)(b)(c_1)$	DPNH-bc_1 complex (*115*)
$(f_S)(b)_2$	succinic-b complex (*118*)
(a)	cytochrome oxidase (*62–65*)

The composition and properties of these derivative particles suggest a few working principles Any particle which lacks cytochrome *a* no longer is capable of reacting with molecular oxygen The removal of cytochrome *a* is relatively easily achieved, and a variety of particles without *a* have been prepared The separation of the two flavoproteins from one another or of cytochromes *b* and c_1 from one another is a much more formidable task which finally has been accomplished Thus there are particles containing either f_S or f_D or both; and particles containing cytochrome *b* or c_1 or both.

Apparently any one oxidation-reduction component can be detached from the electron transport particle independently of the detachment of the other components Thus ETP can be fragmented into a red particle which contains no cytochrome *a* (*116*). In turn the flavoproteins in this red particle can be split out without necessarily affecting the links of the red cytochrome to the particle; or one of the red cytochromes (*b*, c_1, or *c*) can be detached without the other two being involved This suggests that the two flavoproteins and the four cytochromes have independent modes of attachment to the particle But this generalization has to be qualified in the following respects No way has yet been found of detaching cytochromes *b* or c_1 without at the same time detaching cytochrome *a* However, cytochrome *c* can be removed independent of any of the other cytochromes. These observations may be explained in the following terms Cytochrome *c* is present within one of the lipoproteins in the form of a lipid complex (*76*) Various specific methods such as exposure of the particle to phospholipase lead to the complete extraction of cytochrome *c* (*110*) No other cytochrome is bound to the particle in this manner, and thus cytochrome *c* can be extracted quantitatively without the involvement of the other cytochromes The methods for removal of cytochromes *b* or c_1 are ineffective when cytochrome *a* is present in a particle This observation suggests that the links which bind cytochrome *a* to the particle in turn affect the strength of other links which bind cytochromes *b* and c_1.

There is clearly an order in which cytochromes can be detached· *a* always before *b* and c_1, and *b* usually before c_1 At the other end of the chain the flavoproteins can be detached before cytochromes *b*, c_1, and *a*

The electron transfer process is demonstrable even in fragmented particles. Thus in the particle with the composition f_S f_D(b), c_1 *c* the reduction of all the red hemes by succinate or DPNH can be effected, and electrons from these two substrates can be transferred to acceptors such as cytochrome *c*, ferricyanide, or phenazine. Thus, there can be functional connections between the different oxidation-reduction components of a particle which has an incomplete electron transfer chain

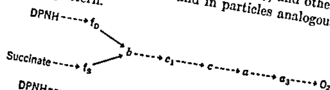
But, depending upon the mode of fragmentation, the connections between the components may or may not be functional Thus in some red particles cytochrome *b* is no longer reducible by either succinate or DPNH while cytochrome c_1 is still functionally linked Electron acceptors such as *ferricyanide or phenazine* can react directly with the flavoproteins in a given particle, but the rate of reaction of a flavoprotein with ferricyanide *(at low levels of electron acceptor) is greater per unit of flavin* when the flavoprotein is attached to a particle The differential in rate may be from three to ten times

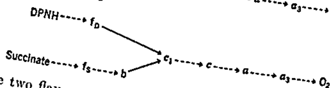
VI. Electron Transfer Chain

In ETP the two flavoproteins and four hemoproteins form a continuous network by which electrons originating from succinate or DPNH are ultimately transferred to molecular oxygen We may refer to the complete system responsible for this process as the electron transfer chain The cytochromes in the chain (*a*, *b*, c_1, and *c*) are fully reducible under anaerobic conditions by either substrate (*26*, *119*). The rate of oxidation of succinate plus DPNH is no faster than that of DPNH alone (the faster of the two substrates) If, as appears to be the case, there are separate entry points for both f_s and f_D, then these two must intercommunicate in some such fashion that any cytochrome which is attached to one spur is reducible by electrons originating from the substrate of the other spur

There are a group of highly selective inhibitors of the electron transfer process which have been of great diagnostic value The reaction with molecular oxygen catalyzed by the terminal cytochrome *a* segment of *the chain is inhibited by cyanide and* H_2S *at low concentrations and by* azide and CO at relatively high concentrations (*41*). These reagents could bind the copper, the *a* heme, or both. Antimycin A, a reagent discovered by Strong *et al* (*120*) and applied for the first time by Potter and Reif (*121*), inhibits the oxidation of succinate or DPNH by cytochrome *c* but not the oxidation of reduced cytochrome *c* by molecular oxygen (*122*) An amount of antimycin A equivalent to the flavin in a given particle preparation can completely inhibit the flow of electrons This is perhaps the most efficient inhibitor yet uncovered in the biochemical literature (*32a*). The precise site of action of this inhibitor is still unknown Sodium amytal is a recent addition to the list of specific inhibitors. Ernster *et al* (*123*) were the first to observe the inhibitory effects of amytal on the oxidation of DPNH at some site beyond the flavoprotein level The oxidation of succinate is not affected by amytal at levels inhibitory for the oxidation of DPNH.

According to Chance (*124*), Slater (*125*), and others, the respiratory chain in heart mitochondria and in particles analogous to ETP has the following pattern.





The two flavoproteins and four cytochromes are arranged in the order assigned in the diagram They are presumed to be in the closest molecular apposition though essentially free molecules. They interact with one another serially by molecular collision. Thus oxidized flavoprotein is reduced by substrate, reduced flavoprotein reacts with oxidized cytochrome *b*, reduced *b* reacts with oxidized cytochrome c_1 etc., etc Chance and Williams (*126*) have recently introduced the concept of restricted rotation for the various components of the chain These are believed to be arranged like a series of contiguous ball bearings Each ball can rotate to one side or to the opposite so that the functional group can either accept electrons from a donor at one side or donate electrons to an acceptor on the opposite side

VII. The Role of Lipid

There are a considerable number of observations which suggest a functional role for lipid in the electron transfer process (*109, 127–129*). Lipid has been found to be intimately associated with f_D (*39*), c_1 (*56*), cytochrome *c* (*76, 110*), $f_s b$ (*132a*), and coenzyme Q The interaction of reduced coenzyme Q with oxygen (*130*), of reduced cytochrome *c* with oxygen (*61b*), of succinate with coenzyme Q (*132a*), and of succinate with cytochrome *c* (*75a, 132b*) require the presence of lipid when particles have been rendered lipid-deficient Cytochrome *c* (*76*) and coenzyme Q appear to be localized in lipid segments of the chain and their role in electron transfer has to be conceived of in terms of oxidoreductions within lipid.

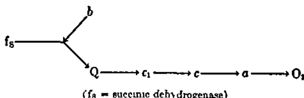
On the basis of these observations it appears reasonable to assume that lipids intervene between any two oxidation-reduction protein components of the electron transfer chain The movement of electrons from one component to the next may proceed through the mediation of substances in

the lipid packets which can serve a shuttle role—accepting electrons from the donor protein and donating electrons to the acceptor protein. Coenzyme Q and lipid cytochrome *c* are at present the only known lipid components which can assume this shuttle role.

Perhaps the significance of lipid may be more clearly recognized when the mechanism of oxidative phosphorylation is elucidated The basic function of the mitochondrion is the coupling of electron transport to oxidative phosphorylation, and it is probable that lipid is a device for providing a built-in, nonaqueous medium in which this coupling can be effected That is to say, the phosphorylated intermediates which presumably are formed in coupled systems may be stable only in a nonaqueous medium, and lipid may be in effect a reaction vessel in which the coupling reactions can proceed in absence of water From this point of view the oxidation-reduction components which are localized in lipid such as coenzyme Q and lipid cytochrome *c* may be of considerable relevance to the mechanism of oxidative phosphorylation

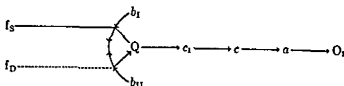
VIII. The Arrangement of the Electron Transfer Chain

Let us consider first the electron transfer chain which intervenes between succinate and DPNH on the one end and molecular oxygen on the other This is in fact the chain of the electron transfer particle (ETP) According to Green *et al* (*132*) the sequence for the succinic chain is the following where arrows indicate the direction of electron flow:



(f_S = succinic dehydrogenase)

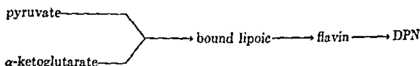
A similar sequence appears to apply to the DPNH chain except that an additional component may intervene between the DPNH dehydrogenase (f_D) and coenzyme Q (indicated by dotted line)



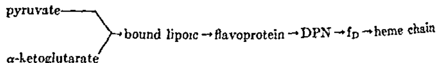
The junction point of f_s and b_I and of f_D and b_{II} may be the nonheme iron which is found in close association with both f_s and f_D. Cytochrome *b* appears to consist of two components: b_I which can be isolated as part of the succinic dehydrogenase in the form of f_sb (*182a*) and b_{II} which is readily detached from both f_s and f_D (*66*). There is both a structural and functional link between f_s and f_D which accounts for the reduction of f_D by succinate and of f_s by DPNH.

The available evidence suggests that cytochrome *b* is not on the main pathway from succinate or DPNH to oxygen and that it may serve merely to conduct electrons from other systems like the fatty acid dehydrogenase systems into the chain The reduction of *b* by succinate or DPNH may merely be a reflection of the fact that f_s, f_D, and *b* join at a common junction point in the chain—perhaps by way of nonheme iron

The segment of the chain between citric cycle substrates (other than succinate) and f_D has yet to be fully clarified According to Koike and Reed (*143b*) the sequence from pyruvate or α-ketoglutarate to DPN is as follows:

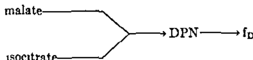


A flavoprotein intervenes between enzyme-bound lipoic acid and DPN. The nature of this flavoprotein is still in doubt According to Massey (*88a,b*) this flavoprotein is specific for the oxidation of reduced lipoic acid (or lipoamide) but it has yet to be demonstrated that bound and free lipoic acid are oxidized by the same flavoprotein. That is to say, there is no assurance that the functional groups responsible for interaction with external thioctamide in the flavoprotein which Massey has isolated is identical with the functional group responsible for interaction with bound lipoic acid in the flavoproteins of the pyruvic and α-ketoglutaric complex. If Massey is correct then the sequence would be:



Two flavoproteins would follow in order. the first for oxidation of reduced lipoic acid by DPN and the second for oxidation of DPNH by some oxidation-reduction component in the electron transfer chain

The two other pyridinoprotein enzymes, malic and isocitric would then require only one flavoprotein, namely f_D.



If there is only one flavoprotein which carries out both the oxidation of reduced lipoic acid (bound) by DPN and the oxidation of DPNH by some component in the chain then it would be necessary to assume that f_D works one way in the oxidation of pyruvate and α-ketoglutarate (as DPN reductase) and another way in the oxidation of malate and isocitrate (as DPNH oxidase) Another consequence of the one flavoprotein hypothesis would be that DPN would not be an obligatory intermediary between f_D and the chain Furthermore, the flavoprotein responsible for the oxidation of reduced lipoic acid may not even interact with DPN, but be linked directly to the electron transfer chain.

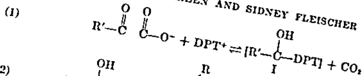
IX. Citric Acid Cycle

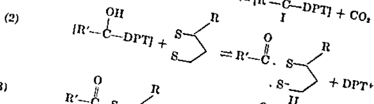
A Oxidations of the Citric Cycle

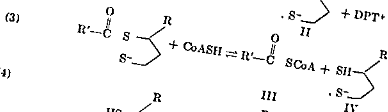
Mitochondria from all sources—animal, plant, and microbial—couple the oxidation of pyruvate and CO_2 to water by way of the citric acid cycle to the synthesis of ATP from ADP and inorganic phosphate The five oxidative steps of the cycle are (1) oxidation of pyruvate to acetyl CoA and CO_2; (2) oxidation of isocitrate to α-ketoglutarate and CO_2; (3) oxidation of α-ketoglutarate to succinyl CoA and CO_2, (4) oxidation of succinate to fumarate, and (5) oxidation of malate to oxalacetate (*2, 133, 134*). Four of the five steps (except step 4) require pyridine nucleotide in the form of DPN or TPN.

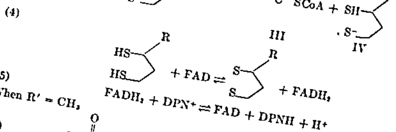
B α-Ketoacid Dehydrogenases

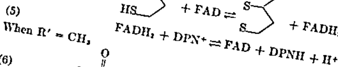
The isolated pyruvic and α-ketoglutaric dehydrogenases are composite proteins with multiple functional groups and activities (*135–138*) The component activities which are identical for the two dehydrogenases may be summarized as follows: (1) the interaction of the ketoacid with diphosphothiamine with formation of the α-hydroxyethyl derivative (I) of diphosphothiamine (in the case of pyruvate) and liberation of CO_2 as shown in Fig 20 (*139–143a*); (2) the interaction of (I) with oxidized lipoic acid to form *S*-acetyl lipoic (II) and diphosphothiamine; (3) the interaction of II and CoASH to form acetyl CoA (III) and dihydrolipoic

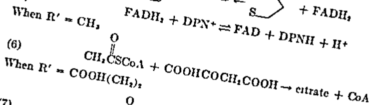


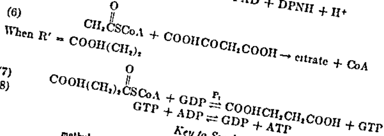


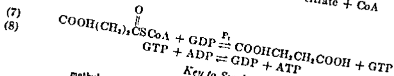


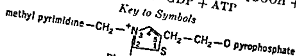


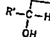












Expanded Formula of I

DPT⁺—Diphosphothiamine

—Oxidized lipoic acid

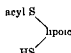
—Reduced lipoic acid

FAD—Flavin adenine dinucleotide
GDP—Guanosine diphosphate

GTP—Guanosine triphosphate

FIG. 20 Formulation of component steps in the oxidative decarboxylation of ...uvic and α-ketoglutaric acids From the data of Gunsalus and Smith (142), Reed ...a), Koike and Reed (143b), Sanadi and Littlefield (135), and Krampitz *et al.* (140)

acid (IV); (4) the oxidation of (IV) by flavin, and (5) the oxidation of reduced flavin by DPN The DPNH formed can then be regenerated via the electron transfer chain The first step is a decarboxylation with formation of an active aldehyde (hydroxyethyl-derivative of diphosphothiamine) The second is an oxidation of the bound aldehyde by reduced lipoic acid to form *S*-acyl lipoic acid



The third is a transacylation from *S*-acyl lipoic to CoASH The fourth is an oxidation of dihydrolipoic to the disulfide ring form (*142*, *143a*), and the last the oxidation of reduced flavin by DPN (*143b*) In the case of α-ketoglutarate the steps are the same except that a succinaldehydo-derivative of diphosphothiamine is first formed which reductively succinylates the ring form of lipoic acid, and finally a succinyl group is transacylated to CoASH with formation of succinyl CoA

Magnesium and diphosphothiamine are involved in the decarboxylation (*144*, *145*), diphosphothiamine and lipoic in the oxidation of aldehyde to an acyl derivative (*140*, *142*, *143a*), lipoic and CoASH in the transacylation (*135*, *136*, *142*, *143a*), and lipoic, DPN and flavin in the terminal oxidation (*142*, *143a*)

Both the pyruvic and α-ketoglutaric dehydrogenases exist as a single protein[4] entity which catalyzes the five separate processes involved in the over-all conversion of the α-ketoacid to the acyl CoA and CO_2 This composite protein has a molecular weight of 4×10^6 in the case of the pyruvic dehydrogenase (pigeon breast muscle) (*147*) and 2×10^6 in the case of the α-ketoglutaric dehydrogenase (pig heart muscle) (*148*) All prosthetic groups but DPN are firmly bound to the protein. Per mole of enzyme protein there is one mole of diphosphothiamine, six moles of lipoic acid and one mole of flavin adenine dinucleotide.

C Isocitric and Malic Dehydrogenases

The protein with which isocitric dehydrogenase is associated catalyzes two consecutive reactions (1) the oxidation of D-isocitrate to oxalo-

[4] The studies of Lannane and Ziegler (*146*) suggest that the pyruvic and α-ketoglutaric dehydrogenase systems are one and the same except that there is a separate carboxylase for each of the two keto acids During isolation one of the two carboxylases may be selectively inactivated, and thus it could appear that two separate protein entities are involved

succinate by TPN (or DPN); and (2) the Mg-catalyzed decarboxylation of oxalosuccinate to α-ketoglutarate and CO_2 (*149*) The oxidation and decarboxylation can be studied independently of one another.

Malic dehydrogenase is the only uncomplicated pyridinoprotein enzyme in the group. It catalyzes the reversible oxidation of L-malate to oxalacetate by DPN (*150*). The equilibrium at pH 7.0 lies in the opposite direction which in effect means that the oxidation of L-malate will be strongly inhibited by the presence of oxalacetate and that malate oxidation can go on maximally only when the product is removed as fast as it is formed

D. Auxiliary Enzymes of the Citric Cycle

Besides the five oxidative steps of the cycle there are two hydrolytic processes which are superimposed on the sequence of oxidations: (1) the reversible hydration of fumarate to L-malate catalyzed by fumarase (*151*, *152*), and (2) the reversible dehydration of citrate to cis-aconitate and the hydration of cis-aconitate to D-isocitrate, catalyzed by aconitase (*153*) Fumarase has no special requirement (*154*), but iron appears to be necessary for the enzymic activity of aconitase (*155*).

E Repetitive Character of the Citric Cycle

The citric cycle of Krebs may be represented as in Fig. 21 and the repetitive attribute of this cycle as in Fig. 22. At the end of each cycl

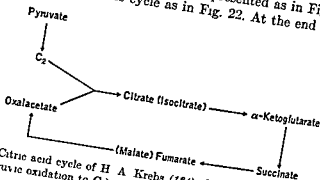


Fig 21 Citric acid cycle of H A Krebs (*134*) C_2 = acetyl CoA (cf Fig 20 for details of pyruvic oxidation to C_2)

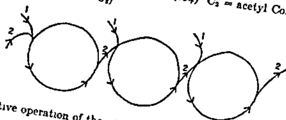


Fig 22 Repetitive operation of the citric acid cycle 1 Acetyl CoA. 2. Oxalacetate

oxalacetate is regenerated, and a condensing partner for another molecule of acetyl CoA is made available Thus the process can continue indefinitely until all available pyruvate has been converted to acetyl CoA.

F. Localization of Citric Cycle Enzymes

When the mitochondrion of heart muscle is mechanically fragmented into submitochondrial particles (ETP or ETP$_H$) citric cycle activity is lost. The malic and isocitric dehydrogenases as well as fumarase and aconitase become solubilized, and parts of the pyruvic and α-ketoglutaric dehydrogenases also become detached in both particulate and soluble forms It is curious that the β-hydroxybutyric dehydrogenase which catalyzes the oxidation of its substrate by DPN remains firmly attached to the particle (*24*) This enzyme is not primarily concerned in the implementation of the citric acid cycle The oxidative enzymes as well as the auxiliary enzymes of the fatty acid cycle are also detached coincident with the fragmentation of mitochondria These results suggest that the pyridinoprotein enzymes of the citric acid cycle and the enzymes of the β-oxidation sequence for fatty acids are each in the form of a complex which is attached to the heme chains of mitochondrial units (*156*). As soon as the mitochondrion is disintegrated the attachment of each of these complexes is weakened, and the complex is liberated in the form either of soluble enzymes or particulates, depending upon the structural idiosyncracies of the individual enzyme proteins The same applies to the bound pyridine nucleotide of mitochondria (DPN and TPN) which is liberated as soon as mitochondrial structure is disrupted (*157–159*). The chemical principles which underlie the association of functionally related enzymes in the form of a complex and which account for the binding of these complexes to the respiratory chain have yet to be elucidated.

G The Mitochondrial Electron Transfer Sequence

To visualize the electron transfer sequence of mitochondria one must add on to the chain of ETP a segment which contains the pyridinoprotein enzymes of the citric acid cycle as well as bound pyridine nucleotide. The entry points to the mitochondrial chain are only certain members of the citric cycle (pyruvate, malate, α-ketoglutarate, and succinate). Neither externally added DPNH nor isocitrate can be oxidized by intact heart mitochondria (*160*) DPNH is not oxidized, presumably because it cannot penetrate into the interior of the metachondrion Isocitrate is not [illegible]

The fatty acid oxidizing enzymes are linked with the heme chain in

a way which is not understood as yet The flavoprotein enzymes (acy dehydrogenases plus the electron transfer flavoprotein) have been founc to interact at least with cytochrome *b* while the pyridinoprotein enzymes (D,β-hydroxyacyl CoA dehydrogenase) may interact with the same bound pyridine nucleotide which serves the other pyridinoprotein enzymes of the citric acid cycle (*113*).

X. Oxidative Phosphorylation

A P/O Ratios

Belitzer and Tsibikova (*161*), and Kalckar (*162, 163*) discovered the phenomenon of oxidative phosphorylation in 1939 It was not until 1948 that it was realized that oxidative phosphorylation is another parameter of the citric acid cycle and electron transport (*2*) During the oxidation of the substrates of any of the pyridinoprotein enzymes, three molecules of inorganic phosphate disappear for each atom of oxygen absorbed, while during the oxidation of succinate only two molecules of phosphate disappear per atom of oxygen (*164, 165*) These ratios are referred to as P/O ratios In point of fact, the P/O ratio of oxidation of α-ketoglutarate is 4 0 (*164, 166*), but one mole of inorganic phosphate is esterified by a process which is not related to oxidative phosphorylation. Succinyl CoA (the product of the oxidation) is converted to phosphoryl CoA, and the phosphoryl group is eventually transferred to ADP through guanosine diphosphate as intermediary (*142, 167, 168*) (cf Fig 20) Phosphoryl CoA does not accumulate as an intermediate being bound to the appropriate enzyme

B Requirement for Oxidative Phosphorylation

Molecular oxygen, substrate, phosphate, magnesium ions, and adenine nucleotide are the only requirements for oxidative phosphorylation (*165*) ADP can be used in an amount equivalent to the inorganic phosphate or in catalytic amounts In the latter case ATP formed in the reaction is allowed to react with glucose in presence of hexokinase (or any other suitable system for regenerating ADP) In a tightly coupled mitochondrial system the oxidation of substrate grinds to a halt as soon as phosphate or ADP is exhausted, and this block can be relieved by addition respectively either of extra phosphate or of a system such as the hexokinase-glucose system which regenerates ADP from ATP (*169*)

Mitochondria contain bound ATP (ADP) which may provide the link between externally added ADP and the phosphorylated compounds or intermediates generated during oxidative phosphorylation (*170, 171*) They also contain a suboptimal amount of magnesium and phosphate ions.

C Uncoupling of Oxidative Phosphorylation

In the presence of reagents such as 2,4-dinitrophenol (*165*, *172*) or gramicidin (*165*) citric cycle oxidations go on at the same or even increased rates, but inorganic phosphate is not esterified to form ADP The known uncoupling agents cover a wide gamut of chemical structure, and it is difficult to believe that these are all acting by the same mechanism For a review of uncoupling agents and their possible mode of action, cf Lehninger (*173*) Uncoupled systems no longer require the presence of added phosphate or ADP for citric cycle oxidation.

The transition from coupled to uncoupled oxidation may take place merely by allowing a mitochondrial suspension to stand for a short time in a hypotonic medium (*174a*) Occasionally recoupling can be achieved by the addition of ATP and other cofactors (*174a*) The hallmark of uncoupling is usually an increased rate of oxidation of succinate (*21*)

D Latent ATPase

When mitochondrial suspensions become uncoupled by dinitrophenol the ATPase activity of the suspension is greatly augmented (a factor of 5 to 15) (*174b*, *175*) In general the rise of ATPase activity parallels the uncoupling of oxidative phosphorylation, but the correlation is not perfect Certainly it cannot account fully for the uncoupling action of 2,4-dinitrophenol (*176*) The ATPase activity of intact and coupled mitochondria is reasonably close to zero (*177*).

E Exchange Reactions during Oxidative Phosphorylation

[illegible]

between water and ATP (*181*, *182*) All these three exchange reactions are very much faster than the rate of oxidative phosphorylation, and none depend upon oxidative reactions These exchange reactions are sensitive to 2,4-dinitrophenol and other uncoupling agents

F Oxidative Phosphorylation in Partial Systems

The known reactions which lead to oxidative phosphorylation are listed in Table II. The coupled oxidation of DPNH is observed only in ETP$_H$ and not in mitochondria (*24*) Lehninger (*183*, *189*) has prepared a submitochondrial particle by exposure of rat liver mitochondria to digitonin This particle catalyzes the coupled oxidation of β-hydroxybutyrate to acetoacetate but has lost the capacity for citric cycle oxida-

tions In animal mitochondrial systems, ferricyanide can replace oxygen as electron acceptor for citric cycle oxidations (*165, 184, 185*), and various electron donors can replace citric cycle substrates (*186*). The coupled oxidation of these complex ions (ferrocyanide and silicomolybdate) requires the presence of divalent metal ions in relatively high concentration and is

TABLE II
P/O VALUES FOR CITRIC CYCLE OXIDATIONS AND PARTIAL SYSTEMS

System	P/O	Reference
Pyruvate + $\frac{5}{2}$ O_2 (over-all)	→3	*183*
DPNH + $\frac{1}{2}$ O_2	2 to 3	*164, 183*
β-Hydroxybutyrate + $\frac{1}{2}$ O_2	→3	*164, 183*
β-Hydroxybutyrate + 2 $Fe_3(CN)_6$	1 6	*183*
Pyruvate + 10 $Fe_3(CN)_6$ (over-all)	1 to 2	*165, 184, 185*
2 Ferrocyanide + $\frac{1}{2}$ O_2	1 8	*186*
Reduced silicomolybdate + $\frac{1}{2}$ O_2	2 6	*186*
2 Reduced cytochrome c + $\frac{1}{2}$ O_2	0 8	*187, 188*

observed only with intact mitochondria (*186*). The usual uncoupling agents have exactly the same effect on these artificial oxidative processes as on citric cycle oxidation.

G Bound Pyridine Nucleotide

Mitochondria contain bound pyridine nucleotide in an amount equivalent to about six times the concentration of flavin (*26, 157*). When mitochondrial suspensions are allowed to age they lose their complement of pyridine nucleotide. This loss can be either reversible or irreversible If reversible, the rate of oxidation is restored by addition of DPN to the original level before aging, and the theoretical P/O ratio obtains If irreversible, the rate of oxidation is restored by DPN, but the additional oxygen uptake induced by added DPN is uncoupled to phosphate esterification Apparently the capacity of an aged mitochondrial suspension to rebind DPN is a critical test of the capacity for oxidative phosphorylation during citric cycle oxidations. It can readily be shown that pyridine nucleotide is actually reincorporated when potentially coupled mitochondria are exposed to pyridine nucleotide (*190, 191*).

H. Multiplicity of Phosphorylation Sites

A P/O ratio of two can be interpreted in several different ways.

(a) two one-electron oxidoreductions each involving one molecule of phosphate

(b) one two-electron oxidoreduction involving two molecules of phosphate

(c) three successive one-electron oxidoreductions [as in (a)] except that the efficiency is only 66⅔% (in this case only one of the paired electrons is involved in phosphorylation)

(d) two successive two-electron oxidoreductions [as in (b)] except that the efficiency is only 50%

Although the first alternative is the only one which is usually taken seriously there is no valid basis at present for ignoring the other possibilities When two electrons from succinate are transferred to molecular oxygen, do the electrons describe separate pathways, and does each pathway contribute to oxidative phosphorylation? If the latter is the case, then in effect the movement of electrons from one donor to the next acceptor in line can lead to a P/O ratio of two since the two electrons undergo coupled oxidoreduction in two separate loci The question of the number of sites at which oxidative phosphorylation can proceed cannot be decided with the limited information now available.

I. Stabilization of Coupling Capacity

There is considerable variation in the stability of the coupling capacity among mitochondria from different sources—liver mitochondria are notoriously unstable whereas heart mitochondria top the list of ruggedness Heart mitochondria can be kept frozen indefinitely without loss of phosphorylative activity (*21*) whereas the same treatment without special precautions leads to rapid deterioration of the phosphorylative activity of liver mitochondria (*20*). The same differential in stability also applies to particles which are derived from the parent mitochondrion by chemical or physical means

There are a few devices or principles for stabilization of coupling capacity which have been recognized Anaerobic conditions protect the phosphorylating properties of mitochondria whereas aerobic conditions accelerate the rate of deterioration (*190, 192*) Versene or substrate duplicate the effect of anaerobic conditions These observations suggest that one condition for stability is the maintenance of one or more components of the electron transfer chain in the reduced state The addition of ATP is another device for stabilization of mitochondria though the effect of ATP is not as striking as that obtained by maintaining the mitochondrion under anaerobic conditions (*193, 194*)

J. The Mechanism of Oxidative Phosphorylation

The phenomenon of oxidative phosphorylation is the linking of phosphate esterification to any of the oxidations of the citric cycle in the respiratory chain Since there are at least 9 oxidoreductions involved in the oxidation of four members of the cycle by molecular oxygen (sub-

strate + pyridine nucleotide, reduced pyridine nucleotide + flavin, reduced thioctic acid + flavin, reduced flavin + iron, reduced b + Q, reduced Q + c_1, reduced c_1 + c, reduced c + a, reduced a + O_2), and since the possibility of even more intermediates is still a strong one, there is a wide choice of oxidoreduction reactions which could be involved in coupling. The pinpointing of the coupling reactions has yet to be accomplished though some are more favored than others As yet not a single oxidoreduction in the electron transfer sequence has been defined sufficiently to permit speculation as to how the coupling is accomplished. For example, in the reduction of pyridine nucleotide by substrate, there is no knowledge of the form and structural disposition of the bound pyridine nucleotide In the interaction between bound DPNH and the flavin of the DPNH dehydrogenase the same uncertainty applies Until we can describe in detail how each of the oxidoreductions of the electron transfer chain takes place, the question of the mechanism of coupling can hardly be considered seriously

There are a few generalizations which can be stated with some confidence even without the detailed knowledge of the mechanism of electron transport (*125*, *126*) The esterification of inorganic phosphate must be a consequence of either of two main possibilities or variations thereof (*126*, *182*, *195*):

(1) Electron donor (A) + electron acceptor (B) → oxidized A + reduced B
Oxidized A + reduced B + phosphate → phosphate ester of oxidized A or reduced B

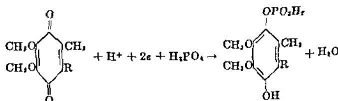
(2) A + B + phosphate → oxidized A + phosphoric ester of reduced B

or

A + B + phosphate → phosphoric ester of oxidized A + reduced B

Either the electron donor and acceptor interact and form a bond which by a displacement reaction leads to a phosphorylated intermediate, or the oxidant (reductant) is phosphorylatively reduced (oxidized). The transfer of the phosphoryl group to ADP is, strictly speaking, a secondary process which we need not consider in the context of the present discussion

Coenzyme Q could conceivably undergo reduction to the monophosphoric ester



According to Clark *et al* (190) the monophosphoric esters of naphthohydroquinone can be oxidized to the corresponding quinone with liberation of inorganic pyrophosphate These authors have, therefore, concluded that the C—O—P bond becomes a transient high-energy bond when the quinol phosphate is oxidized

At the time of writing of this chapter there is no firm experimental evidence in favor of any mechanism of coupled phosphorylation It seems in the cards that the elucidation of mechanism will parallel the accumulation of precise information about the nature of the electron transfer process

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Glycolysis

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I. Introduction

In general the term glycolysis is conventionally used to describe the reaction sequence involved in the anaerobic fragmentation of sugars in the cell In some animal tissues one of the breakdown products that may be formed is lactic acid, in insect muscle it may be α-glycerophosphate; in yeast, ethanol and carbon dioxide are the final products; in bacteria, alcohol or mono- or dicarboxylic acids may accumulate; and in plants under some conditions either ethanol or pyruvic acid is produced.

One of the brilliant chapters of modern biochemistry is that concerned with the description of enzymic reactions responsible for the sequence of events in glycolysis In its history are found the names of the pioneers of biochemistry. These investigators developed the techniques and concepts which are now an integral part of the science and gathered

about them students who are now leaders in the field. To enumerate only a few of these pioneers, Buchner, Harden, Young, Robison, Meyerhof, Neuberg, Embden, Parnas, Needham, the Coris, and Warburg would embrace the names of those who are principal architects of biochemical thought.

No attempt will be made to outline in detail the historical aspects of glycolysis, since excellent reviews are available covering this subject (*1, 2*). The modern era of biochemistry began in 1897 when the Buchners developed cell-free techniques for the analysis of fermentation reactions. This was followed by the elegant experiments of Harden and Young which implicated the phosphate esters (*3*) and prepared the way for the careful studies on fermentation initiated by Meyerhof and others in the 1920's The masterful work of Warburg on triphosphopyridinenucleotide, triosephosphate dehydrogenase, and aldolase led to the development of a methodology which has been adopted by biochemists and applied to broad fields of research. The investigations of the Coris on the problems of polysaccharide synthesis and their successful crystallization of several glycolytic enzymes laid the groundwork for the description of glycolysis in terms of physical-chemical concepts

It will be noted that there have been few new developments affecting the general features of glycolysis as described in the preceding edition of this work Very largely, progress has been a matter of extending research to include many additional species and tissues, refinements of techniques, consolidation of earlier developments, purification of enzymes, and new reaction techniques.

There has grown up a sharp dichotomy of "glycolysis" versus "other pathways of carbohydrate metabolism" (see Chapter 5) This division has flowed naturally from the early successes of biochemists in explaining the anaerobic formation of carbon dioxide from glucose by yeast and lactic acid by muscle It is convenient and proper to talk of pathways but it is often misleading to speak of enzymes belonging to certain pathways Thus a cell confronted with a glucose molecule may phosphorylate it and then oxidize it to phosphogluconate, or it may isomerize it to fructose-6-phosphate In one case it is destined for glycolysis, in the second for oxidation. It is simply a matter of one's point of view as to whether the phosphorylating enzyme should be placed in the glycolytic or oxidative scheme. Since the latter scheme arrived late (historically) on the biochemical scene, hexokinase is dubbed a glycolytic enzyme In short, while there is good justification in tracing out the stepping stones which constitute a metabolic pathway there is not always justification in arbitrarily assigning a given enzyme exclusively to one pathway or another.

II. Enzymes of the Glycolytic Cycle

For more than three decades the enzymes of the glycolytic cycle have been the subject of vigorous research by many investigators. Since much has been written concerning the details of the glycolytic reactions, this

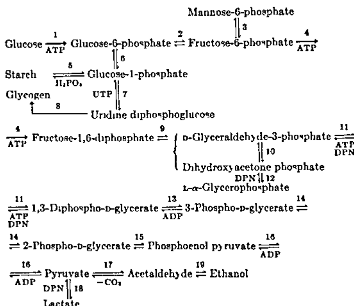


FIG. 1. Reactions of the Embden-Meyerhof Scheme. The individual reactions are catalyzed by the following enzymes: *1* Hexokinase, *2* Phosphoglucoisomerase, *3* Phosphomannoisomerase, *4* Phosphohexokinase, *5* Phosphorylase, *6* Phosphoglucomutase, *7* Uridine diphosphoglucose pyrophosphorylase, *8* Glycogen uridine diphosphoglucose transglycosylase, *9* Aldolase, *10* Triosephosphate isomerase, *11* Glyceraldehyde phosphate dehydrogenase, *12* α-Glycerophosphate dehydrogenase, *13* Phosphoglyceryl kinase, *14* Phosphoglyceryl mutase, *15* Enolase, *16* Pyruvate kinase, *17* Pyruvate carboxylase, *18* Lactic dehydrogenase, *19* Alcohol dehydrogenase.

section will discuss only the highlights of the many enzymes involved (Sections A,1–A,3). A discussion of the enzymes involved in polysaccharide transformations is found in Chapter 6. The cycle is depicted in Fig. 1.

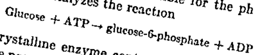
A. Hexokinases

All cells which utilize free sugars contain a group of enzymes called the hexokinases which have the property of catalyzing the transfer of

phosphate from ATP to suitable monosaccharide acceptors to form the corresponding phosphorylated intermediates Within the general group of hexokinases are found enzymes of both low and high specificities All require the participation of ATP as the phosphate donor and a metal, usually magnesium The number of known hexokinases continues to grow as may be well imagined from the almost universal occurrence of glucose throughout the living kingdom

1. *Yeast Hexokinase*

In 1927 Meyerhof made the important observation that aged extracts of skeletal muscle would ferment glucose only after the addition of a preparation obtained from autolyzed yeast (*4*). Further work on this observation soon indicated that the factor which was absent in the aged muscle extract was the enzyme responsible for the phosphorylation of glucose The enzyme catalyzes the reaction



$$\text{Glucose} + \text{ATP} \rightarrow \text{glucose-6-phosphate} + \text{ADP}$$

Although the crystalline enzyme contains only one protein component yeast hexokinase possesses a rather broad specificity, since it transfers th terminal phosphate group of ATP to glucose (*5*, *6*), fructose, glucosamine (*7*), and mannose The relative rates of phosphorylation of these sugars depend to a great extent on their concentrations in the reaction mixture At low sugar concentrations, glucose and mannose react rapidly, while fructose is phosphorylated slowly, conversely, at high sugar concentrations fructose is phosphorylated more rapidly than the other three sugars These differences are related to the difference of their Michaelis constants (K_m) with hexokinase The K_m value for glucose is 1.5×10^{-4}; for mannose, 1.0×10^{-4}; for fructose, 1.5×10^{-3}, and for glucosamine, 1×10^{-4} mole per liter.

There is strong evidence that the more chemically reactive form of fructose, β-fructofuranose, is the actual substrate, rather than the fructopyranose (*8*) D-Galactose, D-xylose, L-rhamnose, sucrose, maltose, lactose, trehalose, and raffinose are inert as substrates

When yeast hexokinase is saturated with glucose, the turnover number (TN) is 13,000 molecules of glucose phosphorylated per enzyme molecule per minute, when saturated with fructose, 26,000; with glucosamine, 12,000, and with mannose, 6,000. When these sugars are added simultaneously to the enzyme system, the total activity is far less than would be expected if a summation of individual activities were occurring A competitive inhibition may therefore be observed between the four sugars for the active center of the enzyme, the degree of inhibition being a function of the concentration of sugar. These results support the con-

clusion that one enzyme protein rather than a complex of closely associated enzymes is responsible for the phosphorylation of these sugars (*8*).

Biotin-deficient yeast ferments glucose and fructose more slowly than when growth occurs with optimum amounts of biotin present (*9*) Such cells respond to added biotin by increased production of carbon dioxide The effect of the biotin action is upon the hexokinase as can be shown by the low activity in biotin-deficient cells (*10*) and by the increase in this activity which the addition of this enzyme produces in the cell-free extract (*11*) However it has not yet been possible to show a complete dependence of hexokinase on biotin

Yeast hexokinase is not an exception to the general rule that kinases make and break P-O bonds rather than C-O bonds (*12*)

2 *Plant Hexokinase*

Enzyme activity which resembles yeast hexokinase in respect to substrate specificity, reaction products, and metal requirements, is found in a number of plants, both in soluble and particulate form (*13*) There is a specific fructokinase in peas in addition to a relatively nonspecific hexokinase which utilizes both glucose and fructose (*14*)

While Mg^{++} and Mn^{++} are commonly found to fulfill the cationic requirements of the enzyme it is interesting that Zn^{++} appears essential for the elaboration of hexokinase by *Neurospora* and may constitute a portion of the enzyme (*15*) It has not, however, been shown that this enzyme does not also require Mg^{++}

3 *Mammalian Hexokinases*

In the mammals, too, hexokinases both soluble (*16*) and particulate (*17, 18*) are known Here it has not been possible to achieve the purification attained with yeast

a BRAIN HEXOKINASE A particulate preparation has been obtained which is free of interfering enzymes and has, therefore, been amenable to rather extensive characterization with respect to its specificity, which resembles that of yeast hexokinase (*19*) A number of sugars serve as substrates Relative maximum rates and K_m values for some are shown: fructose (1 4, 1 6 $\times$ 10^{-3} M), glucose (1 0, 8 $\times$ 10^{-6} M), deoxyglucose (1 0, 2 7 $\times$ 10^{-5} M), 1,5-sorbitan (1 0, 3 $\times$ 10^{-3} M) and mannose (0 5, 5 $\times$ 10^{-6} M) The enzyme contains essential sulfhydryl groups.

b FRUCTOKINASE. Crude muscle hexokinase phosphorylates both glucose and fructose (*16*) The two activities may be separated with ammonium sulfate Crude extracts have a fructose: glucose ratio of 0 26; on fractionation with ammonium sulfate between 0.35 and 0.54 saturation, a fructose: glucose ratio of 0 148 is observed Muscle fructokinase

is not inhibited by glucose and is saturated by its substrate at unusually high concentrations The reaction product is not the expected fructose-6-phosphate but rather fructose-1-phosphate. Muscle glucokinase has not as yet been examined to any extent for its specificity.

Rat liver extracts also contain two highly specific kinases, namely glucokinase and fructokinase (*20, 21*). Glucokinase, in the presence of ATP and Mg^{++}, forms glucose-6-phosphate; fructokinase catalyzes the formation of fructose-1-phosphate Beef liver fructokinase, like rat liver fructokinase, is strongly activated by potassium chloride (*22*) Sodium and ammonium ions are relatively inert. The affinity of the enzyme for fructose is very high, the K_m being lower than 5×10^{-4} moles per liter. This is in contrast to muscle fructokinase, which has a very weak affinity for its substrate

Beef liver fructokinase is unusually sensitive to ADP which acts as a competitive inhibitor It is also inhibited by ATP when in excess of the Mg^{++} present *Ideal assay conditions are provided when ATP is generated* by some feeder system such as creatine phosphate and ATP-creatine transphosphorylase. The sensitivity of the enzyme to the ATP/Mg^{++} ratio suggests a site for control of this enzyme by alterations in the concentrations of these substances by physiological processes (*23*).

c. Other Mammalian Hexokinases. Calf heart muscle yields both a soluble and a particulate hexokinase which appear to differ in their properties (*24*) The partial purification of hexokinases from heart, skeletal muscle, and liver has been described (*25*).

A survey of glucokinase activity in different muscles from different mammals indicates a variation in specific activity (*26*) Thus, in the rabbit the sequence of glucokinase activity is heart muscle > skeletal muscle > stomach muscle > diaphragm muscle, the ratio of activities being 1 5 1·0 5 0 2 In the rat the ratio of heart to skeletal muscle activity is 1.4, in the guinea pig, 1.6, and in the chicken, 1.9. Extracts of skeletal muscle of rat and man are significantly more active than those obtained from similar muscles of the guinea pig, rabbit, and chicken

d. The Role of Hexokinase in Active Transport of Glucose The active transport of glucose across the intestinal membrane or into cells of various other tissues has been explained in the past by postulating that the glucose was specifically phosphorylated by a hexokinase, then transported and dephosphorylated. While it has not yet been possible to provide an adequate explanation of this selective absorption it has been possible to show that the hexokinase theory is not tenable. Thus 6-deoxyglucose is also actively absorbed but it obviously cannot be phosphorylated in the number 6 position (*27*) Similarly 3-methylglucose is also *readily absorbed although it is not a substrate for hexokinase* (*28*). Indeed

the phosphorylative ability of the intestinal wall *vis a vis* specific sugars and the ability of the intestine to absorb them are by no means parallel (*29*).

B. Phosphoglucomutase

Shortly after glucose-1-phosphate was discovered as the product of phosphorolysis of glycogen, Colowick and Cori observed an enzyme in extracts of muscle, liver, brain, kidney, and yeast which converted the acid-labile phosphate of glucose-1-phosphate to an acid-stable phosphate identified as glucose-6-phosphate (*30, 31*) The enzyme which catalyzes this conversion, phosphoglucomutase, has been isolated in crystalline form by subjecting water extracts of rabbit skeletal muscle to a series of heat treatments and ammonium sulfate fractionations (*32*) This enzyme makes up about 2% of the water-soluble protein of muscle The turnover number per 10^5 gm of protein is about 16,800 moles of glucose-1-phosphate converted to glucose-6-phosphate per minute.

The enzyme is inhibited by fluoride in the absence of inorganic phosphate By varying the concentration of the three reactants, magnesium fluoride, and glucose-1-phosphate, it was shown that a complex of $(Mg)(F)^2$(organic phosphate) was formed. The equation,

$$(Mg)(F)^2(\text{glucose-1-phosphate}) \times \frac{\%\ \text{residual activity}}{\%\ \text{inhibited activity}} = K = 0.17$$

describes the stoichiometry of the reaction (*32*).

Some confusion existed as to the precise mechanism involved in the shift of phosphate from the 1- to the 6-position of glucose In 1938 Kendal and Stickland (*33*) reported activation of phosphoglucomutase by the addition of fructose-1,6-diphosphate Later, on the basis of the observation that phosphoglucomutase contains nondialyzable phosphate which exchanges with P^{32} of glucose-1-phosphate, it was proposed that the enzyme protein combined either with glucose-1-phosphate or glucose-6-phosphate to form a cyclic substrate-enzyme complex (*34*) The complex could split either at the 1- or the 6-position to yield glucose-6- or glucose-1-phosphate, respectively This proposal did not explain the Kendal and Stickland effect, nor did it account for the frequent observation that the same enzyme would vary considerably in activity depending on the batch of substrate used in the assay system The complete and unequivocal solution to the problem was presented by Leloir and his co-workers (*35*) They showed that phosphoglucomutase required an extremely active coenzyme which was found as an impurity in fructose diphosphate, in synthetic glucose-1-phosphate (0 to 0 3%), and in enzymically prepared glucose-1-phosphate (0 1%) The coenzyme was soon identified as

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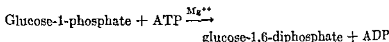
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glucose-1,6-diphosphate, which, in contrast to fructose diphosphate, is alkali-stable, acid-labile It forms a barium-insoluble salt, is a nonreducing ester, and yields on acid hydrolysis an equivalent of inorganic phosphate and glucose-6-phosphate (*35*) The coenzyme has been synthesized by known synthetic procedures and has been identified as α-D-glucose-1,6-diphosphate. The coenzyme saturates the enzyme protein at a remarkably low concentration, the K_m being 4×10^{-7} moles per liter.

Living cells have two methods available for the biosynthesis of this cofactor:

1. Yeast, muscle, and plants



$$\text{Glucose-1-phosphate} + \text{ATP} \xrightarrow{Mg^{++}} \text{glucose-1,6-diphosphate} + \text{ADP}$$

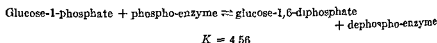
2 Escherichia coli



$$2\ \text{Glucose-1-phosphate} \xrightarrow{Mg^{++}} \text{glucose-1,6-diphosphate} + \text{glucose}$$

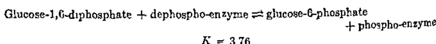
The cofactor has been found in varying amounts in all living tissue.

Active phosphoglucomutase (of rabbit muscle origin) exists as a phospho-enzyme containing one atom of P per enzyme molecule (*36*). In this form it is capable of converting both glucose-1-phosphate and glucose-6-phosphate to the 1,6-diphosphate When an excess of acceptor is present the enzyme is converted to the dephospho form. The equilibrium constants of the following reactions have been measured directly (*37*)



$$\text{Glucose-1-phosphate} + \text{phospho-enzyme} \rightleftharpoons \text{glucose-1,6-diphosphate} + \text{dephospho-enzyme}$$

$$K = 4\ 56$$



$$\text{Glucose-1,6-diphosphate} + \text{dephospho-enzyme} \rightleftharpoons \text{glucose-6-phosphate} + \text{phospho-enzyme}$$

$$K = 3\ 76$$

On the basis of this information it can be computed that ΔF^0 *for the* hydrolysis of the phospho-enzyme is $-3{,}900$ cal This value is intermediate between the values for glucose-6-phosphate and glucose-1-phosphate and implies a moderate phosphate potential The phosphate is bound to the enzyme through a primary hydroxyl oxygen, being found in phosphoseryl peptides after the enzyme is subjected to mild hydrolysis Although the value $-3{,}900$ cal is for the reaction between the un-ionized reactants, the pK_2 of the free and bound phosphate are probably sufficiently similar so that $-\Delta F^0$ is a good approximation for $-\Delta F'$ at pH 7 0

A fascinating example of the simplicity in design in nature is suggested by the observation that the phospho-enzyme carries the phosphate

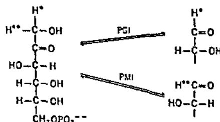
on a serine residue (*38*) and that the phosphoserine appears in amino acid sequences similar to those found with the phosphorylated (and diisopropylphosphorylated) chymotrypsin, thrombin, trypsin, and cholinesterase (*39*, *40*) The analogy is carried even further by direct and indirect evidence that phosphoglucomutase activity, like chymotrypsin activity, depends also on a histidine moiety (*41*) While at first thought it is surprising to find that such apparently diverse enzymes function through a common mechanism, consideration of the net changes involved in the bond-splitting of an acyl compound and a phosphoryl compound shows a formal similarity

C PHOSPHOHEXOISOMERASE

In 1933 Lohmann (*42*) discovered in skeletal muscle the enzyme responsible for the reversible conversion of glucose-6-phosphate to fructose-6-phosphate The equilibrium mixture contains 70% glucose-6-phosphate and 30% fructose-6-phosphate

In addition to the specific phosphoglucoisomerase, a phosphomannoisomerase has been isolated from rabbit muscle (*43*) This enzyme is distinct from phosphoglucoisomerase from which it can be separated by ammonium sulfate fractionation and by use of alumina It can also be distinguished from the first enzyme by the considerably lower pH at which it is active (*44*).

The "intimate" mechanisms of phosphoglucoisomerase (PGI) and phosphomannoisomerase (PMI) actions have been revealed with the aid of deuterium-tracer experiments (*45*) Although both enzymes have a common substrate, fructose-6-phosphate, they each activate a different one of the two hydrogens bound to C-1 This is readily proven by the



observation that glucose-6-phosphate-1-D does not exchange its deuterium for hydrogen on being treated with the phosphoglucoisomerase in ordinary water, but it does so when both enzymes are present. It can also be shown by permitting the enzymic interconversion of glucose-6-phosphate and fructose-6-phosphate in deuterium oxide that no inter- or

intra-molecular migration of a hydride ion occurs It is suggested that the intermediate in the case of one enzyme must be a *cis*-enediol and a *trans*-enediol in the other.

It is interesting that the metabolic block imposed on glucose utilization by administering 2-deoxyglucose to eviscerated animals has been traced to the inhibitory action of its phosphorylation product, 2-deoxyglucose-6-phosphate, on phosphoglucoisomerase (*46*)

Phosphoglucoisomerase has been used in the preparation of glucose-6-phosphate from the relatively inexpensive barium fructose-6-phosphate The isomerization is carried out in the presence of a small amount of seed crystals of the insoluble barium glucose-6-phosphate · 7 H_2O (*47*)

Apparently phosphomannose isomerase whether from rabbit muscle or erythrocytes shows a requirement for divalent cations and is inhibited by organo-mercurials Phosphoglucoisomerase does not show a metal requirement Because the enzyme is inhibited by ethylenediaminetetraacetic acid it is possible to assay phosphoglucoisomerase in a mixture of the two enzymes It is interesting that two such functionally similar enzymes should differ in their need for cations (*48a*)

D PHOSPHOFRUCTOKINASE

In 1909 it was observed that a stable, phosphorylated form of fructose, fructose-1,6-diphosphate, accumulated when glucose was fermented by cell-free preparations of yeast (*48b*). In 1936 it was shown that a specific enzyme was responsible for the formation of the diphosphate and could be found in yeast and muscle extracts (*49*) This enzyme, phosphofructokinase, has since been highly purified and its properties studied (*50*) This enzyme catalyzes the transfer of the terminal group of ATP to fructose-6-phosphate in the presence of Mg^{++} Its specificity is high, since it does not catalyze any other transfer reaction.

By subjecting extracts of rabbit muscle to a series of ammonium sulfate fractionations, a cut was obtained which had a turnover number of about 1400 per minute per 10^5 gm of protein. Since the enzyme is unusually labile to acid conditions in all purification procedures the pH had to be held above 7 There is evidence that the stability of the enzyme is affected not only by the pH, but also by the nature of the ionic environment and concentration Thus, certain anions such as oxalate, pyrophosphate, phosphate, and sulfate tend to stabilize buffered solutions of the enzyme (*50*).

In addition to the usual 6-phosphofructokinase found in various organisms, 1-phosphofructokinase has been obtained from muscle and liver (*51*).

The 6-phosphofructokinase found in rabbit muscle can utilize ITP

and UTP almost as well as ATP, even after it has been rendered free of nucleoside diphosphokinases (*52*). It can be confidently expected that most crude preparations of kinases will utilize triphosphonucleotides other than ATP but conclusions regarding the specificity of such enzymes must be reserve until it is shown that internucleotide phosphorylation is not occurring

E. Aldolase

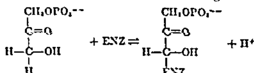
In 1934 dialyzed extracts of muscle and yeast were found to catalyze the cleavage of fructose diphosphate to dihydroxyacetone phosphate (*53*). A closer examination of this reaction revealed that instead of catalyzing the formation of dihydroxyacetone phosphate, the enzyme split fructose diphosphate to equal amounts of dihydroxyacetone phosphate and D-3-phosphoglyceraldehyde. This conclusion was based on the observations that (1) in the process of purifying aldolase, triosephosphate isomerase is removed and hence triosephosphates accumulate with no further change, and (2) trapping agents, such as cyanide, hydrazine, and sulfite, will effectively combine with the triose phosphates and thereby fix the primary reaction products (*31*)

The enzyme has been crystallized and its physical properties studied in some detail (*54*)

Muscle aldolase is strongly inhibited by traces of heavy metals and its activity is not decreased by metal binding reagents such as cysteine and α-α'-dipyridyl Yeast aldolase, which has been extensively purified by Warburg, is inactivated by cysteine and reactivated by ferrous, zinc, or cobaltous ion (*55*) Aldolase of *Clostridium perfringens*, on the other hand, is reactivated by ferrous or cobaltous ions in the presence of cysteine (*56*) Pea aldolase is not inhibited by heavy metals nor by cysteine and is not activated by ferrous or cobaltous ions (*57*).

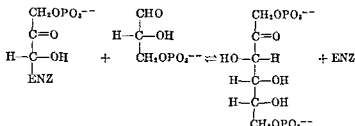
While a number of aldehydes will replace D-glyceraldehyde-3-phosphate in the aldolase-mediated condensation the specificity for dihydroxyacetone phosphate is absolute It is not surprising therefore that a transient enzyme-dihydroxyacetone phosphate complex is apparently formed when the enzyme is presented with dihydroxyacetone phosphate or with fructose-1,6-diphosphate (*58–60*) Direct spectrophotometric evidence for this complex also exists (*61*)

The mechanism of aldolase action here is envisaged as follows:



If tritiated water is present, one atom of T is introduced per molecule of dihydroxyacetone phosphate. If D-glyceraldehyde-3-phosphate is also present, fructose-1,6-diphosphate is formed, but it is unlabeled. The most reasonable explanation for this is that the enzyme displaces a specific hydrogen atom of the two hydrogen atoms bound to C-3.

The over-all reaction is then as follows.



An important consequence of the specific affinity which the enzyme has for one partner of the aldol condensation is that it permits preferential exchange of the C-4, C-5, C-6 portion of fructose-1,6-diphosphate with D-3-phosphoglyceraldehyde (*62*) It is thus possible to obtain unsymmetrical labeling of glucose in biological systems even though only the enzymes of the Embden-Meyerhof system are involved

The previous belief that there was an absolute requirement for the hydroxyl groups in the 3-4 position to be *trans* has been modified by the demonstration that D-tagatose-6-phosphate which differs from the fructose isomer in having the opposite configuration at C-4, can be split (*63*) However, there is a strong preference for the *trans* configuration

The broad specificity which aldolase exhibits with regard to the aldehyde partner in the aldol condensation permits the formation of sedoheptulose-1-phosphate from dihydroxyacetone phosphate and D-erythrose with the yeast or muscle enzyme (*64, 65*) If D-erythrose-phosphate is used, then the 1,7-diphosphate can be obtained (*66, 67*).

The existence of a specific fructose-1-phosphate aldolase in rat liver has been proposed on the grounds that, unlike rabbit muscle aldolase, the liver enzyme catalyzes the following reaction (*68*):



Fructose-1-phosphate ⇌ D-glyceraldehyde + dihydroxyacetone phosphate

The enzyme, termed fructose-1-phosphate aldolase, was however found to attack fructose-1,6-diphosphate (*69*) A similar preparation from rabbit liver possessing activity against both substrates has been shown to undergo a relative loss in its fructose-1,6-diphosphate aldolase

activity in the course of its purification, lending support to the idea that there may indeed be an aldolase distinct from the classic one derived from muscle (*70*) On the other hand beef liver has yielded a crystalline enzyme which attacks both esters and otherwise resembles muscle aldolase in its specificity (*71*)

Indeed, during the course of the purification of the beef liver enzyme, variable contamination with fructose diphosphate could have led to an apparent shift in the relative activities of fructose-1,6-diphosphate aldolase and fructose-1-phosphate aldolase (*71*)

While it has been claimed that a specific enzyme in rat liver, distinct from diphosphofructoaldolase, condenses formaldehyde with dihydroxyacetone phosphate to yield D-1-phosphoerythrulose (*72*), there is no question that rabbit muscle aldolase catalyzes the same reaction. The beef liver enzyme also catalyzes this reaction (*71*).

Rabbit muscle aldolase apparently contains all of its cysteine in the reduced form (28 moles per molecule of enzyme) (*73*) The 10 most reactive sulfhydryl groups can be reacted with p-mercuribenzoate without decreasing enzyme activity Further combination of sulfhydryl groups with this reagent now causes the activity to diminish but the presence of substrate does not prevent this decrease It has been suggested that the sulfhydryl groups are needed to maintain the structural characteristics essential to the activity of the enzyme, but that they are not essential in the sense that they are involved in the primary catalytic act

A remarkable heat stable aldolase has been isolated from *Bacillus stearothermophilus* which undergoes very little inactivation when held at 70° for 40 minutes, but shows an enhanced heat sensitivity if pretreated with a sulfhydryl compound such as cysteine (*74*).

F Triosephosphate Isomerase

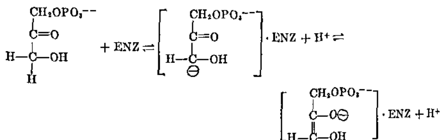
In their studies on the aldolase reaction, Meyerhof and Kiessling (*75*) observed the catalytic effect of this enzyme, which is described by the reaction:



Its role in glycolysis is obvious, since it is responsible for the equilibration between the two triose units derived from the cleavage of fructose diphosphate by aldolase The enzyme occurs in large amounts in skeletal muscle as well as in malignant tumors, brain, and yeast.

When dihydroxyacetone phosphate is incubated with triosephosphate isomerase in tritium oxide almost one atom of tritium is stably bound by the equilibrium product (*59*, *76*). The product when treated with the

enzyme in H_2O loses its tritium. The postulated mechanism is as follows



A specific one of the two hydrogens bound by the carbinol carbon is labilized. It is interesting that this is not the same hydrogen which is labilized by the action of aldolase. Hence it is concluded that the enediol intermediates in the two cases must be stereoisomers of each other.

Triosephosphate isomerase which has been crystallized from calf muscle in a 50% yield has the remarkably high turnover number of 2×10^6 per 10^5 g (77).

G. Phosphoglyceraldehyde Dehydrogenase (PGAD)

The reaction catalyzed by this enzyme is particularly interesting because it couples phosphorylation to dehydrogenation, producing an ester with a high $-\Delta F^0$ of hydrolysis. Coupling may be carried out by at least four different systems. (1) in yeast, the alcohol dehydrogenase system; (2) in animal tissues, primarily the lactic dehydrogenase system; (3) under some conditions, the reduction of dihydroxyacetone phosphate by glycerophosphate dehydrogenase, and (4) the utilization of reduced DPN by the general pool of metabolic reactions.

Much interest exists as to the mechanism of the reaction.

For some time it has been known that the sulfhydryl groups of mammalian PGAD had to be maintained in the fully reduced state for enzyme activity. The oxidized form could be readily transformed to the reduced state by exposure to suitable SH reagents such as glutathione and cysteine. The reduced form of the enzyme, having free SH groups, is irreversibly inhibited by low concentrations of iodoacetic acid (78). Yeast PGAD is, however, less sensitive to inhibition by iodoacetic acid and is also less susceptible to inhibition by oxidation.

Some years ago crystalline mammalian PGAD became readily available (79). It was then noticed that when phosphoglyceraldehyde was added to a solution of crystalline rabbit muscle PGAD, a 340-mμ absorption peak, characteristic of reduced DPN, appeared in the absorption spectrum of the enzyme protein (80). In addition, adenine, nicotinamide,

and ribose were isolated from the hydrolytic products of the crystalline protein It soon became apparent that two molecules of DPN were tightly bound to the protein Although DPN could not be removed by exhaustive dialysis nor by repeated recrystallizations, DPN could be split off the molecule either by exposure to a phosphatase or to norite (*80*) It is of interest to note that the DPN-free protein could not be recrystallized unless DPN were added to the solution The recrystallized product again contained two molecules of DPN per molecule of enzyme protein The yeast enzyme also has a strong affinity for DPN but may be freed from it by recrystallization

This enzyme was the first dehydrogenase found to have a pyridinonucleotide strongly attached to the protein Most other pyridinonucleotide dehydrogenases have a more freely dissociable system

The enzyme was found to possess a remarkable multifunctional activity (*81*), catalyzing the following reactions

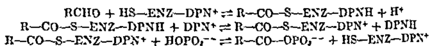
a Oxidation of phosphoglyceraldehyde
b Acetaldehyde + phosphate $\xrightarrow{\text{DPN}}$ acetyl phosphate
c Acetyl phosphate + phosphate^{32} $\rightleftharpoons$ acetyl phosphate^{32} + phosphate
d Acetyl phosphate + arsenate → acetate + arsenate + phosphate
e Acetyl phosphate + GSH $\rightleftharpoons$ acetyl GSH + phosphate
f Acetyl phosphate + CoA $\rightleftharpoons$ acetyl-CoA + phosphate

It is noteworthy that regardless of the nature of the reactions, DPN is an essential component In DPN-free systems no exchange reactions are observed

Two equivalents of DPN are released when two equivalents of *p*-chloromercuribenzoic acid are added to the crystalline enzyme (*82, 83*). The SH reagent, possessing a higher affinity for the SH groups of the protein, displaces bound DPN, which is presumably attached to the protein by two SH bridges. A minute dissociation does occur, however, since on exposure of the intact crystalline protein to radioactive DPN, a rather rapid incorporation of radioactivity onto the enzyme surface is observed (*84*) The apparent dissociation constant has been estimated to be about 10^{-7} moles per liter for the mammalian enzyme and about 10^{-4} moles per liter for the yeast enzyme-DPN complex Racker and Krimsky suggested that the carrier of these important SH groups in the protein molecule is glutathione (*85, 86*)

The binding of reduced glutathione to muscle phosphoglyceraldehyde dehydrogenase has been confirmed (*87*), but it has not been possible to verify the role of this compound as a sulfhydryl carrier and it may be that the previously observed correlation of activity to quantity of glutathione bound was fortuitous

If the reaction proceeds by the P-independent attachment of the aldehyde to the enzyme followed by the P-independent oxidation to the acyl form as shown, then the initial rate of DPN reduction should be the same with or without P. The enzyme has such a high turnover rate that it is technically difficult to measure the rate when stoichiometrically equivalent amounts of enzyme and substrate are employed. The problem has been solved by using D-glyceraldehyde as the substrate since this compound is oxidized at a much slower rate. Kinetic studies made with the substrate have given results which are in accord with the proposed mechanism. In addition, the acyl-enzyme compound can be formed and isolated when 1,3-diphosphoglycerate reacts with the DPN-free enzyme. This enzyme-acyl complex reacts with DPNH to yield the aldehyde and DPN. Further proof of the mechanism shown is provided by the agreement of the stoichiometry of the P-independent reaction with the number of reactive sites



$$RCHO + HS\text{—}ENZ\text{—}DPN^+ \rightleftharpoons R\text{—}CO\text{—}S\text{—}ENZ\text{—}DPNH + H^+$$
$$R\text{—}CO\text{—}S\text{—}ENZ\text{—}DPNH + DPN^+ \rightleftharpoons R\text{—}CO\text{—}S\text{—}ENZ\text{—}DPN^+ + DPNH$$
$$R\text{—}CO\text{—}S\text{—}ENZ\text{—}DPN^+ + HOPO_3^{--} \rightleftharpoons R\text{—}CO\text{—}OPO_3^{--} + HS\text{—}ENZ\text{—}DPN^+$$

This interpretation has been criticized by Warburg and his co-workers on the ground that no action occurs in the absence of inorganic phosphate when DPN is provided only in the enzyme-bound form (88). It is postulated that the external DPN may introduce inorganic phosphate

By the aid of O^{18}-labeled tracer it has been established that the cleavage of the acyl phosphate bond involves the *C—O* rather than the P—O bond (89). The well known ability of arsenate to replace phosphate in [illegible] has been explained in the past on the [illegible] ility. [illegible] hown that the acetate released from acetyl phosphate during the [illegible] lysis catalyzed by this enzyme acquires O^{18} (90) Hence an ester containing covalently bound arsenate must be formed.

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Both yeast and rabbit muscle 3-D-phosphoglyceraldehyde dehydrogenases transfer H to the same site of the pyridine ring when they reduce DPN (β-side) (92)

H. Phosphoglyceryl Kinase

It has been known for sometime that in the oxidation of phosphoglyceraldehyde to phosphoglyceric acid, ADP is required. Closer inspec-

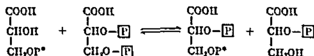
tion indicated that two independent enzyme systems are involved, one being triosephosphate dehydrogenase which catalyzes the formation of 1,3-diphosphoglyceric acid, and the other a specific enzyme responsible for the transfer of the phosphate of 1,3-diphosphoglyceric acid to ADP. Adenylic acid is inert except in the presence of adenylic kinase.

The enzyme has been purified and crystallized from brewer's yeast by Bücher (*93*) and from human muscle by Kubowitz and Ott (*94*). The reaction rates are very high, the forward rate being 320,000 moles per minute per mole of enzyme and the back reaction 36,400. The enzyme is highly specific and will not react with 2,3-diphosphoglyceric acid or with phosphoenolpyruvate

I Phosphoglyceromutase

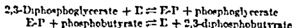
Both 3-phosphoglyceric acid and 2-phosphoglyceric acid are readily converted to pyruvic acid by cell-free extracts of yeast, bacterial, plant, and animal tissue Phosphoglyceromutase is the specific enzyme responsible for catalyzing the equilibrium between these two acids

An interesting but seemingly irrelevant observation was made by Greenwald (*95*) in 1925 when he isolated D-2,3-diphosphoglyceric acid from erythrocytes Sutherland and co-workers (*96*) demonstrated that the Greenwald ester was the counterpart of glucose diphosphate in the conversion of 3-phosphoglyceric to the 2-isomer. Thus when P^{32}-labeled 3-phosphoglyceric acid was added to the enzyme and nonlabeled diphosphoglyceric acid, the radioactivity was soon distributed between the substrate, the coenzyme, and the reaction product The reaction may be depicted as follows

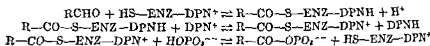


The K_m for 2,3-diphosphoglyceric acid is of the order of 10^{-8} moles per liter in contrast to the glucosediphosphate system, which has a K_m of 4×10^{-7}

Although the mechanism of action of phosphoglyceromutase would be expected by analogy to be quite similar to that of phosphoglucomutase, it remains to be established that a phosphoprotein intermediate exists. On one hand the enzyme is reported to catalyze the formation of diphosphobutyrate from 2,3-diphosphoglycerate and 2,3-dihydroxybutyrate via the following mechanism (*97*):


2,3-Diphosphoglycerate + E ⇌ E-P + phosphoglycerate
E-P + phosphobutyrate ⇌ E + 2,3-diphosphobutyrate

If the reaction proceeds by the P-independent attachment of the aldehyde to the enzyme followed by the P-independent oxidation to the acyl form as shown, then the initial rate of DPN reduction should be the same with or without P. The enzyme has such a high turnover rate that it is technically difficult to measure the rate when stoichiometrically equivalent amounts of enzyme and substrate are employed The problem has been solved by using D-glyceraldehyde as the substrate since this compound is oxidized at a much slower rate. Kinetic studies made with the substrate have given results which are in accord with the proposed mechanism In addition, the acyl-enzyme compound can be formed and isolated when 1,3-diphosphoglycerate reacts with the DPN-free enzyme. This enzyme-acyl complex reacts with DPNH to yield the aldehyde and DPN. Further proof of the mechanism shown is provided by the agreement of the stoichiometry of the P-independent reaction with the number of reactive sites



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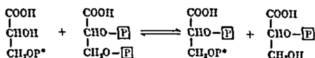
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I PHOSPHOGLYCEROMUTASE

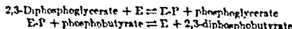
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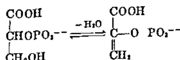

2,3-Diphosphoglycerate + E ⇌ E-P + phosphoglycerate
E-P + phosphobutyrate ⇌ E + 2,3-diphosphobutyrate

On the other hand the yeast enzyme, which has been crystallized (*98*), while it contains bound phosphate, does not phosphorylate the 2- or 3-phosphoglycerate (*99*). Oddly enough the incubation of 2,3-diphosphoglycerate with the enzyme leads to the formation of 2- and 3-monophosphoglycerate and inorganic phosphate. Apparently even the crystalline enzyme is contaminated with diphosphoglycerate phosphatase

The corresponding enzyme from rabbit muscle has been purified to the point where mole for mole it has about the same activity as the yeast enzyme, it has a molecular weight of 64,000 compared to 112,000 for the yeast enzyme (*99*)

J. Enolase

Enolase catalyzes the removal of a molecule of water from 2-phosphoglyceric acid as follows:



The product has now a considerably higher value for $-\Delta F^0$ of hydrolysis

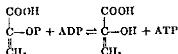
The enzyme has been crystallized as the mercury salt from yeast. Both yeast (*100*) and the animal enzyme (*101*), as well as the plant system (*102*, *103*), require Mg^{++}, Mn^{++}, or Zn^{++} for activity In the presence of Mg^{++} and phosphate, fluoride exerts a strong inhibitory effect on the enzyme A magnesium fluorophosphate complex is established, thereby effectively removing Mg^{++} from the reaction mixture.

Enolase is specific only for 2-D-phosphoglycerate and phosphoenolpyruvate of a number of substances tested. Only the completely ionized forms are reactive (*104*) From the nature of the pH effect on K_m it is assumed that the active center contains an ionizable group with a pK of 7 5 Of the three possibilities, sulfhydryl, imidazole and α-amino groups, a sulfhydryl group is ruled out by the indifference of the enzyme to —SH reactive reagents On the other hand, diisopropyl fluorophosphate, which inhibits other enzymes which are believed to have an essential imidazole (histidine in trypsin, chymotrypsin, cholinesterase, and phosphoglucomutase), completely inactivates enolase It has not yet been established whether the inhibitor phosphorylates a serine group in enolase, as it does in the other enzymes.

suitable for activation with a pK of about

K. PYRUVATE KINASE

This enzyme, obtained in crystalline form from human and rat muscle, catalyzes a transfer of phosphate from phosphoenolpyruvic acid to ADP



The enzyme from rabbit muscle can also use IDP, GDP, UDP, and CDP as acceptors (*105*)

The TN is about 6000 per 10^5 gm of protein for the forward reaction and, remarkably, only 12 for the back reaction (*101*)

For many years the transfer from phosphoenolpyruvate to ADP was regarded as an irreversible step in the direction of ATP synthesis Because of this conclusion, many elaborate schemes were devised to circumvent this block in the synthesis of sugars from pyruvic acid. However, in 1945 it was shown that the enzyme is completely reversible providing K^+ or NH_4^+, as well as Mg^{++} and ATP, were added (*106*) In the absence of K^+, the reaction was negligible It was later shown that K^+, NH_4^+ or Rb^+ were essential for the forward reaction (*107*)

L. ANCILLARY ENZYMES

1 *Glucose-6-phosphatase*

The enzymic cleavage of glucose-6-phosphate is a vital step in the conversion of liver glycogen to blood glucose. While liver homogenate has a high potential for catalyzing the cleavage of glucose, the large number of nonspecific (or perhaps better, nonspecified) enzymes which it contains has always made insecure the claims that an enzyme specific for this reaction actually exists in the mixture. However, it now seems reasonable to postulate that such an enzyme resides in the microsomes, even if there is still some reason to question its absolute specificity (*108, 109*) Even the well washed particles contain at least two additional phosphatase enzymes as can be adduced from studies of pH optima, pH sensitivity, and differential effect of inhibitors (*110*).

The enzyme which can hydrolyze nitrophenyl phosphate also catalyzes the transfer of phosphate from this substrate to methanol (*110*). This enzyme, when acting on glucose-6-phosphate, appears to be inhibited by glucose because glucose serves as a good acceptor of phosphate (*111*). This is shown by adding labeled glucose to the reaction mixture; the decrease in liberation of inorganic phosphate is directly proportional to the amount

of labeled glucose incorporated into glucose-6-phosphate. The sensitivity of the enzyme to glucose may thus provide a route to the homeostatic control of glucogenesis. Microsomal glucose-6-phosphatase also attacks glucosamine-6-phosphate (*112*)

There is only one well authenticated instance of an acid phosphatase which splits glucose-1-phosphate where the possibility of explaining the result on the basis of phosphoglucomutase and glucose-6-phosphatase has been eliminated Silkworm blood hydrolyzes both glucose-1-phosphate and galactose-1-phosphate but has no action on glucose-6-phosphate (*113*) It may be conjectured that if the function of glucose-6-phosphatase is to convert glucose-6-phosphate to glucose for circulatory distribution, that glucose-1-phosphatase would serve just as well.

The previous observations that hepatic glucose-6-phosphatase also attacks glucose-1-phosphate is explained by the contamination of the enzyme with phosphoglucomutase, which exists as a soluble cytoplasmic enzyme in liver (*114*).

It has been suggested that glucose-6-phosphatase may be concerned in diabetes because its level rises in the disease but returns to normal when insulin is given (*115*, *116*)

The oral anti-diabetic drug, 1-butyl-3-(*p*-tolylsulfonyl)-urea ("Orinase") inhibits glucose-6-phosphatase in rat liver, *in vitro*. It also inhibits phosphoglucoisomerase. Two other enzymes for which glucose-6-phosphate is also a substrate, phosphoglucomutase and glucose-6-phosphate dehydrogenase, are not affected (*117*).

2. *Fructose Diphosphatase*

Preparations of liver and kidney contain an alkaline phosphatase which will hydrolyze only fructose diphosphate to fructose-6-phosphate (*118*). It requires Mg^{++} for activity and is inhibited by fluoride (see also Chapter 5)

3 *Glycerophosphate Dehydrogenases*

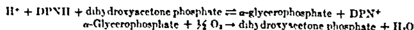
Two different types of enzymes catalyze the reduction of dihydroxyacetone phosphate to L-α-glycerophosphate. The first enzyme requires DPN as the cofactor and has been crystallized from extracts of muscle tissue (*119*) The TN is 26,000 per 10^5 gm of enzyme protein per minute at 20° and at pH 7 0 The equilibrium constant greatly favors the reduction of dihydroxyacetone phosphate·

$$K = \frac{(\text{glycerophosphate})(\text{DPN}^+)}{(\text{dihydroxyacetone phosphate})(\text{DPNH})} = 1\ 4 \times 10^4 \text{ at } 22° \text{ and pH } 7$$

The crystalline protein shows a significant absorption band at 260 mμ suggesting that this dehydrogenase may contain bound DPN similarly to muscle triosephosphate dehydrogenase

The second type of α-glycerophosphate dehydrogenase was first described by Green (*120*) as a particulate system which was coupled to cytochrome *c* This particulate system has now been solubilized by treatment with sodium deoxycholate (*121*) On further purification the enzyme was separated from triosephosphate dehydrogenase, isomerase, and catalase activities and no longer was capable of reducing cytochrome *c* It also did not react with DPN, TPN, or FAD, although it readily reduced suitable dyes The reaction product was dihydroxyacetone phosphate

In mammalian muscular tissues, as is well known, the presence of lactic acid dehydrogenase provides a fermentative reservoir of DPN when the drastic demands of muscular activity exceeds the capacity of the tissue to carry pyruvate on to its oxidation product. Another means for achieving this end is also present in such tissue, namely, α-glycerophosphate dehydrogenase, which can regenerate DPNH from DPN^+. Although mammalian skeletal muscle is abundantly endowed with this enzyme, physiological studies indicate that the lactic acid route actually takes care of the peak load demand In insects on the other hand, the content of lactic acid dehydrogenase appears to be low (*122*, *123*) and α-glycerophosphate plays a key role in gearing energy requirements to glycolysis The cytoplasm of the insect muscle contains abundant dehydrogenase which oxidizes DPNH at the expense of dihydroxyacetone phosphate Interestingly enough, the α-glycerophosphate, so formed, is then reoxidized at the expense of oxygen by the α-glycerophosphate oxidase, which is present in the mitochondria (sarcosomes) (*124*, *125*).



$$H^+ + DPNH + \text{dihydroxyacetone phosphate} \rightleftharpoons \alpha\text{-glycerophosphate} + DPN^+$$
$$\alpha\text{-Glycerophosphate} + \tfrac{1}{2} O_2 \rightarrow \text{dihydroxyacetone phosphate} + H_2O$$

The net result is that DPNH is oxidized by atmospheric oxygen, but an intermediate production of DPN^+ can be maintained at the expense of carbohydrate

4 *ATPases and Adenylic Kinases*

a MYOSIN ATPASE For some time the close association of ATPase and myosin in muscle has suggested an interaction of ATP breakdown with a concomitant change in the myosin structure Well washed myosin will consistently catalyze the cleavage of ATP → ADP + P with a pronounced reduction in the asymmetry of the myosin particles (*126*).

b MUSCLE ATPASE An unstable Mg^{++}-activated ATPase has been

isolated from muscles free of myosin and actomyosin associated with particles (127) Unlike myosin ATI strongly inhibited by Ca^{++} and is strongly activated of its high activity it probably plays a major role in centration of ATP in muscle

c. BRAIN ATPASE. This enzyme is associated with and has been concentrated by high-speed centrifugati

d MITOCHONDRIAL ATPASE. When freshly pr chondria are tested, little if any ATPase activity can ever, when these mitochondrial preparations are allo increase in ATPase activity is consistently observe effect can be abolished by the early addition of one of tides to the reaction mixture It is suggested that the tides may interact by maintaining or resynthesizing i in the mitochondria. Since the sudden increase in results from aging, it is of interest to note that an equ phosphorylative capacity of the mitochondria also occ

The specific ATPase of liver mitochondria has be adenylic kinase This ATPase appears to be associ particles and is activated by Mg^{++}. ATP is the spec ADP serves as a potent inhibitor. The complete dep ATP by aged mitochondria is apparently the result action of the specific ATPase and adenylic kinases

e MUSCLE ADENYLIC KINASE This unusual enzy reaction 2 ADP $\rightleftharpoons$ ATP + AMP (130). It is a heat- enzyme, requiring Mg^{++} for maximum activity It i adenine nucleotides and reaches an equilibrium when added ADP has been converted to the mixture of AM enzyme, originally known as myokinase, also bears the transphosphorylase. It has been crystallized from rabbi It has a moderately low molecular weight of 21,000. T ber, on this basis, is about 1,000,000 (131) Systems ca reaction have been found in a variety of tissues Heat-u inhibited kinases have been found in liver mitochondria muscle, kidney, and brain homogenates (128, 132). In (133), and plant extracts (134) similar systems have be

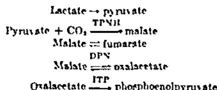
M PREPARATION AND PURIFICATION OF ENZYMES

Excellent directions for the preparation and purifica the glycolytic enzymes may be found in the compreh edited by Colowick and Kaplan (135b). Detailed and ve

the periodically appearing series *Biochemical Preparations*. A remarkable paper has appeared which describes in most welcome detail the preparation of aldolase, phosphoglyceraldehyde dehydrogenase, lactic acid dehydrogenase, glycerophosphate dehydrogenase and pyruvate kinase, all in crystalline form, from rabbit muscle in one procedure (*135c*)

III. Reversal of Glycolysis

It is well known that a large variety of compounds are converted to glycogen by the intact body Fructose, mannose, galactose, and glycerol are among the active precursors Noncarbohydrate sources such as the lower and higher fatty acids serve as effective intermediates The amino acids glycine, alanine, serine, threonine, valine, glutamic acid, aspartic acid, tyrosine, histidine, arginine, citrulline, cysteine, and isoleucine are degraded to products which then may enter the glycolytic cycle Even CO_2 may enter the carbohydrate molecule As may be seen below any substance which can give rise to acetate, pyruvate or Krebs cycle intermediates is a potential source of carbohydrate carbon Particular interest has centered on the conversion of acetate and pyruvate to carbohydrate. Although the pyruvate kinase reaction is reversible it does not participate to an appreciable extent in the synthesis of glycogen from pyruvate in liver (*135a–137*) Pyruvate (and lactate) are inducted into hexose by way of the "dicarboxylate shuttle" (*138, 139*) In muscle (rat diaphragm)



pyruvate is not as readily converted to glycogen as it is in liver However the small amount of conversion that does occur goes largely by reversal of the pyruvate kinase reaction (*140*). Thus pyruvate-2-C^{14} yields 2,5-labeled glucose (in glycogen) in the rat diaphragm whereas in liver the labeled carbon atoms are extensively randomized.

The incorporation of acetate carbon into hexose is readily explained by its conversion to acetyl CoA and subsequent condensation with oxalacetate The participation of the Krebs cycle acids (see Chapter 4) or the glyoxylate shunt (*141*) (see Chapter 4) provides malate or oxalacetate If the glyoxylate shunt is operative a net production of carbohydrate is to be expected

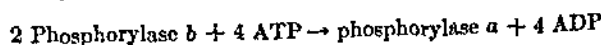
The incorporation of the various carbons of pyruvate into carbohydrate has been compared in several plant tissues (*142*). All positions can contribute to labeling in the sugar formed from pyruvate The order of effectiveness is C-3 > C-2 > C-1. The utilization of C-1 is explained most readily by postulating that this carbon is liberated as CO_2 by the oxidative decarboxylation of pyruvate and fixed into a C_4 acid The remaining C_2 moiety may then be incorporated as above via Krebs cycle enzymes and, if present, by the glyoxylate shunt enzymes, to malate (*139*) For instance, castor bean endosperm (a typical example of an oil reserve tissue) which possesses the glyoxylate shunt system (*141*) is remarkably effective in converting the α and β carbons of pyruvate to hexose As may be expected, pyruvate-3-C^{14} leads to hexose only lightly labeled in C-3, C-4 while with pyruvate-1-C^{14} the label is mainly in C-3, C-4.

IV. *Factors Influencing Glycolysis*

A. Action of Phosphorylase

In animals the energy requirements of metabolism are largely met within the tissues concerned by catabolic reactions of carbohydrates. The regulation of the processes to keep them in pace with metabolic needs is a challenging problem in molecular biology. Because the major storage form of carbohydrate is glycogen much attention has centered on phosphorylase activity, on the grounds that this may be the rate-limiting step A characteristic property of animal phosphorylase strongly suggests that this is indeed the key enzyme through which regulation is exercised. This property is the capacity of phosphorylase to undergo reversible inactivation under the action of enzymes which exist in the same tissues with phosphorylase

Thus in the case of rabbit muscle phosphorylase two well defined forms of the enzyme are obtained from the tissue The first, phosphorylase *a*, is active as obtained; the second, phosphorylase *b*, is derived directly by a halving of the first, and is inactive. The latter may be reactivated by 5′-AMP but the quantity of free nucleotide in the tissue is negligible so that this reaction is physiologically unimportant (*143*). Its physiological activation is probably brought about by the action of phosphorylase *b* kinase and ATP (*144*) This latter reaction results in the regeneration of the original dimer. Because the enzymes involved in the reaction are reasonably pure, reliable stoichiometric data is available, which is in accord with the following equation, with 4 phosphate groups being taken up by the phosphorylase *a* (*145*).

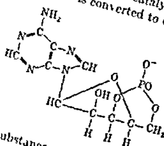


2 Phosphorylase *b* + 4 ATP → phosphorylase *a* + 4 ADP

In the case of dog liver phosphorylase, inactivation occurs by the action of dephosphorylating enzyme, but the molecular weight of the enzyme is not appreciably altered (*146–148*) Reactivation occurs here also by the action of a kinase

It has been long known that epinephrine is glycogenolytic If one accepts the hypothesis that phosphorylase plays the key role one is not surprised to find that epinephrine both in experiments with intact animals and isolated tissue increases the proportion of active phosphorylase (*149*) Even liver slices and homogenates respond to epinephrine by an enhanced production of active enzyme (*148*) Glucagon, the hyperglycemic glycogenolytic factor of pancreas, now available in crystalline form, resembles epinephrine in its action on the liver system but has no effect on the muscle system Adrenal cortical tissue responds only to the adrenocorticotropic hormone (*150*)

The response of the dog liver homogenate to hormones has opened an approach to the problem of the molecular basis of hormonal action Thus is appears that the stimulation of phosphorylase activation, presumably a kinase reaction, by epinephrine or glucagon can be divided into two phases In the first, which is catalyzed by the particulate fraction of liver homogenate, ATP is converted to cyclic 3′,5′-AMP (*151–153*)



Cyclic 3′,5′-adenylic acid

It is this substance which then stimulates the kinase reaction in the supernatant from the homogenate The effect of the hormones is to promote the formation of the cyclic nucleotide To some extent NaF and caffeine also have a stimulatory effect, apparently because they inhibit the degradation of ATP and the cyclic nucleotide, respectively. It is intriguing that similar preparations of heart skeletal muscle and brain also produce cyclic 3′,5′-AMP and respond to epinephrine. Both tissue slices and homogenates of beef adrenal cortex produce the cyclic nucleotide (*154*) Thus far, response to adrenocorticotropic hormone has been found only in the slices

The evidence which has accumulated in favor of the hypothesis that regulation of carbohydrate utilization is achieved through phosphorylase activation and deactivation by the processes discussed is provocative

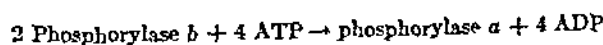
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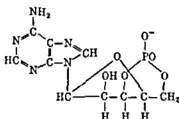


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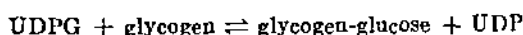
The evidence which has accumulated in favor of the hypothesis that regulation of carbohydrate utilization is achieved through phosphorylase activation and deactivation by the processes discussed is provocative.

However, there is much that must be taken into account and one cannot assume that the problem is almost solved While it is true that resting muscle has a low proportion of phosphorylase in the active form, and it is also true that stimulation to activity increases this proportion, the quantities present when glycogenolysis is not taking place appear to be far too great This still appears to be true after adopting measures designed to prevent the reversion of phosphorylase *b* to *a* What must not be ignored is that phosphorylase catalyzes the formation of glycogen as well as its breakdown Other regulatory factors which play a role in the *in vivo* situation, both in liver and in muscle, are concentration of K^+ and Na^+, pH and availability of P (*143*).

Fascinating and totally unexpected is the discovery that the muscle enzyme contains as an essential prosthetic group, pyridoxal phosphate, a coenzyme hitherto known only in connection with reactions involving amino acids or their corresponding carbonyl analogs (*155, 156*).

Present evidence indicates that the pyridoxal phosphate is bound as an aldamine derivative Reduction of the aldamine derivative with sodium borohydride converts it to a pyridoxylamine derivative which is stable enough to withstand proteinase and acid digestion. ϵ-*N*-Pyridoxyllysine is isolated following such treatment (*157*).

In considering the mechanism of glycogenesis, glycogenolysis, and possible regulatory controls, it is also necessary to reckon with the discovery that glycogen may arise by a uridine diphosphate glucose-requiring reaction, which occurs in liver (*158*).



$$\text{UDPG} + \text{glycogen} \rightleftharpoons \text{glycogen-glucose} + \text{UDP}$$

A similar reaction has been shown to be catalyzed by rat diaphragm (*159*)

B. Pasteur Effect

In cells which normally carry out glycolysis the presence of oxygen usually markedly curtails the utilization of glucose This phenomenon, known as the Pasteur effect, was first observed by Pasteur with yeast cells One of the early explanations proposed, namely the specific poisoning of the (fermentative) glycolytic mechanism has not been convincingly supported by experiment It seems far more likely that the effect results from an interaction of the glycolytic pathway and the oxidative reaction carried out by the mitochondria Both systems are dependent on common substrates, ADP, ATP, and P. Apparently under physiological conditions the aerobic system associated with mitochondria has a greater affinity or greater accessibility for the nucleotides Studies with a reconstructed system in which a glycolytic system is mixed with varying quantities of mitochondria give strong support to this view (*160*).

Further evidence for this reciprocal relation is adduced from the fact that ascites tumor cells normally utilize glucose to a much smaller degree than would be expected on the basis of their content of the glycolytic enzymes The activity of the intact cells begins to approach the potential level when an uncoupler, such as dinitrophenol, is added to suppress the mitochondrial-mediated impoverishment in P (*161, 162*)

The true situation in the cells is complicated not only by the common dependence of these two systems but is further complicated by the operation of the TPN-mediated oxidation of glucose Studies designed to evaluate the role of all three systems, by using appropriate inhibitors, confirm the complexity of the system and suggest competition for the adenine nucleotide as an important factor

The common dependence of the glycolytic and mitochondrial systems has as its consequence the reciprocal of the Pasteur effect, the Crabtree effect (*163*), in which enhancement of the glucose supply suppresses the mitochondrial oxidations (*164*) The modern elegant spectrophotometric techniques of Chance have permitted a kinetic description, on a time scale of seconds, of the result of adding glucose to a suspension of ascites cells The results provide good proof that aerobiosis depletes the extra-mitochondrial glycolytic system of ADP, a view which has long been advanced but has been difficult to prove (*165*)

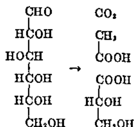
It is obvious that a number of factors may potentially influence the reciprocal relationship between the two processes and may therefore play a role in the Pasteur or Crabtree effects In seeking to explain the effects of various factors which influence glycolysis, such as hormones, cations, etc , it is clear that the primary effect need not be on glycolysis

The precise solution to the problem is not merely an academic matter. A seriously practical aspect of this problem concerns the implication that deranged oxidation with compensatory high aerobic fermentation is a characteristic which distinguishes the cancerous from the normal cell (*166*)

V. Comparative Biochemistry of Glycolysis

Insofar as "glycolysis" refers to the utilization of sugar by the Embden-Meyerhof pathway there is no important difference in principle in the operation of this pathway in the various species The participation of some alternative pathways is discussed in Chapter 5 Continuing surveys show that the conventional pathway is widely distributed throughout the living kingdoms This is not to say that this pathway is the exclusive means by which organisms utilize glucose nor that it cannot coexist with other systems, but in general it is more surprising than not to find organisms (especially nonbacterial) in which this system is defec-

tive. There are of course such cases, and one of particular interest concerns the heterofermentative lactic acid organism, *Leuconostoc mesenteroides*, which converts glucose into CO_2, acetate, and lactate as shown:



Leuconostoc mesenteroides lacks aldolase (*167*) Homofermentative lactic acid bacteria contain aldolase and normally produce two molecules of lactic acid from glucose The generalization that the homofermenters all contain aldolase while the heterofermenters do not, has been established for a number of lactobacilli (*168*)

Where the glycolytic system is present in the various species, differences of course may be found in the nature of the primary hexose source, e g free glucose, starch, glycogen, etc , or in the nature of the ultimate reduced product, e g. lactate or ethanol Moreover the system *in vivo* is not isolated from other systems with which it has intermediates and coenzymes in common The challenge in the future lies in the elucidation of the mechanisms of metabolic control.

Acknowledgment

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CHAPTER 4

The Tricarboxylic Acid Cycle

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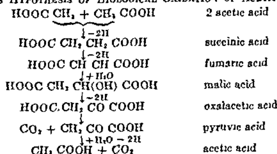
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I. Introduction

The main pathway of the oxidation of foodstuffs in animal tissues is now known to involve two major stages. In the first stage the various substrates undergo a series of changes which result in the formation either of "active acetic acid" or of an intermediate of the tricarboxylic acid cycle. The second or "terminal" stage is common to all foodstuffs and is represented by the tricarboxylic acid cycle. The reactions of the first stage by which the substrate molecule is prepared for entry into the tricarboxylic acid cycle vary from substrate to substrate.

Studies of the pathway of oxidations in animal tissues were initiated about forty years ago by Thunberg (*1, 2*) and Batelli and Stern (*3*), who examined systematically the oxidizability of organic substances in animal tissues A large number of substances—Thunberg lists some forty compounds—were found to undergo oxidation on addition to tissue preparations. In most cases, however, the rate of oxidation was very slow; retrospectively, the most remarkable finding was the observation that among the oxidizable substances succinate, fumarate, malate, and citrate stand out because of the high rate of oxidation and, as Batelli and Stern (*3*) pointed out, because of the similarity of the characteristics of their oxidation to those of the main respiratory processes

The first coherent chemical concept, based on information on the oxidizability of organic substrates, was proposed by Thunberg (*2*) and accepted and supported by Knoop (*4*) and Wieland (*5*). It envisaged the oxidation and condensation of two molecules of acetate to succinate and the oxidation of the latter, via fumarate, malate, oxalacetate, and pyruvate to one molecule of acetate (Scheme 1) Its weakness was the


SCHEME 1
THUNBERG'S HYPOTHESIS OF BIOLOGICAL OXIDATION OF ACETIC ACID (1920)

$HOOC\,CH_3 + CH_3\,COOH$	2 acetic acid
↓ $-2H$	
$HOOC\,CH_2\,CH_2\,COOH$	succinic acid
↓ $-2H$	
$HOOC\,CH\,CH\,COOH$	fumaric acid
↓ $+H_2O$	
$HOOC\,CH_2\,CH(OH)\,COOH$	malic acid
↓ $-2H$	
$HOOC.CH_2\,CO\,COOH$	oxalacetic acid
↓	
$CO_2 + CH_3\,CO\,COOH$	pyruvic acid
↓ $+H_2O - 2H$	
$CH_3\,COOH + CO_2$	acetic acid

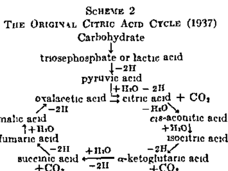
lack of any evidence in support of occurrence of the first step in biological material Toenniessen and Brinkmann (*6*) modified the scheme by sug-

gesting a primary condensation of two molecules of pyruvate to 1,4-diketoadipic acid, but like its predecessor, the key reaction of this scheme lacked experimental support, and was later (7) shown to be improbable by the demonstration that diketoadipic acid is not readily metabolized

The tricarboxylic acid cycle was first proposed in 1937 under the name "citric acid cycle" to describe the intermediary stages of oxidation of carbohydrate in pigeon breast muscle (8) The original scheme as shown in Scheme 2, has subsequently received a number of elaborations It

SCHEME 2

THE ORIGINAL CITRIC ACID CYCLE (1937)



has been possible, in particular, to elucidate the reactions leading from oxalacetic and pyruvic acids to citric acid and from α-ketoglutarate to succinate But in all essential aspects the original scheme still stands

II. The Cycle in Animal Tissues

A THE EVIDENCE IN SUPPORT OF THE OCCURRENCE OF THE CYCLE IN MUSCLE TISSUE

The evidence in support of the cycle in muscle tissue, in particular in pigeon breast muscle, may be dealt with under three headings.

First, all the individual stages which constitute the cycle have been demonstrated to occur in muscle tissue, and the rates at which the individual reactions can proceed are sufficient to account for the maximum rate of respiration The occurrence of some of the reactions in muscle tissue, as already mentioned, has been known since 1911, when Batelli and Stern (3) demonstrated the rapid oxidation of citrate, succinate, fumarate, and malate in frog muscle. In 1936 the work of Martius and Knoop (9, 10) revealed the mechanism of the conversion of citrate into succinate, and the last major step of the cycle was discovered in 1937, when the formation of citrate from oxalacetate and pyruvate was demonstrated (8) It was this reaction which made a series of reactions

into a cyclic sequence, and which linked the series of reactions leading from citrate to oxalacetate with carbohydrate metabolism.

It may be argued that the mere occurrence of a set of oxidative reactions in a tissue does not necessarily mean that these reactions have any connection with the main respiratory processes. This argument would carry weight if the tissue were capable of oxidizing a large variety of substances at rapid rates But this is not the case. The series of reactions *composing the cycle is, in fact,* the only known set of rapid reactions in muscle by which CO_2 and water, the main end products of respiration, can be formed at significant rates from organic substances If one further

TABLE I
CATALYTIC EFFECT OF FUMARATE ON THE RESPIRATION OF MINCED PIGEON BREAST MUSCLE (*12*)

Concentration of added fumarate	Average increase in O_2 uptake observed (μl)	Amount of O_2 calculated for complete oxidation of added fumarate (μl)
0 0001 *M*	151	20
0 0002 *M*	235	40

considers that living cells do not usually possess highly active enzymes unless they employ them in their normal metabolic processes, it becomes very suggestive that the oxidation of the organic acids is part of the main respiratory process in muscle—a view already expressed by Batelli and Stern in 1911 (*3*)

A second set of observations supporting the cycle concerns the catalytic action of the di- and tricarboxylic acids on muscle respiration Such catalytic effects, i e , a stimulation of respiration in excess of any stoichiometric reactions of the added catalyst, were first suggested in 1935 by experiments of Szent-Gyorgyi (*11*), and were conclusively demonstrated in 1936 by Stare and Baumann (*12*) An example demonstrating the effect is given in Table I Similar effects occur with citric acid and in fact with all other members of the cycle (*8*)

These catalytic effects are satisfactorily explained by the concept of the tricarboxylic acid cycle Thus when pyruvate is oxidized via the *cycle, oxalacetate is required for the formation* of citrate For each molecule of oxalacetate which is utilized in *this reaction* another is regenerated in the course of the cycle. The effect of oxalacetate can therefore be expected to be catalytic.

A third series of experiments in support of the cycle (*13*) rests on the use of a highly specific inhibitor, malonate, which in low *concentrations*

(below 0 01 *M*) interferes with one step of the cycle, and one step only, viz, the oxidation of succinate to fumarate This high specificity makes malonate a most valuable tool If a complex metabolic process is found to be inhibited by low concentrations of malonate, it is highly probable that succinic dehydrogenase is a component of this complex process This probability becomes a certainty if it is found that succinate accumulates in the system after the addition of malonate. In work on intermediary metabolism it is always important in establishing the specificity of an inhibitor to demonstrate an accumulation of the substrate in the presence of the inhibitor It has been claimed (*14*) that pyrophosphate is a specific inhibitor of succinic dehydrogenase, but no succinate accumulates when this inhibitor is added to respiring muscle homogenates; thus, the action of this inhibitor cannot be explained by an effect on succinic dehydrogenase only

There are two experiments with malonate which are relevant here (*13*) First, malonate inhibits oxidations in muscle tissue and causes an accumulation of succinate, the inhibition by 0 01 *M* malonate being of the order of 90% Second, this inhibition is abolished by any of the substances which are intermediates in the cyclic scheme But the effect of the addition of these substances to the malonate-poisoned tissue differs from the effect observed in the absence of malonate. As already mentioned, the effect of small quantities of these intermediates is catalytic when they are added in the absence of malonate In the presence of malonate, the effect is no longer catalytic but results in a stoichiometric reaction This leads to the formation of approximately one molecule of succinate per molecule of substrate added, and the O_2 uptake and CO_2 output are of the order expected from the cycle The best examined case is the reaction of fumarate in the malonate poisoned tissue, and the following over-all effect has been experimentally verified (*13*):



$$\text{Fumarate} + \text{pyruvate} + 2\,O_2 = \text{succinate} + 3\,CO_2 + H_2O$$

This equation is expected if the cycle proceeds once from the stage of fumarate through malate, oxalacetate, the tricarboxylic acids, and α-ketoglutarate to that of succinate and stops there. Analogous experiments have been carried out with malate, oxalacetate, citrate, and α-ketoglutarate, and in each case the restoration of respiration is a temporary one It comes to an end when all the added substrate has been converted into succinate, except when rather large quantities of substrates are added In this case, as in the case of the addition of large quantities of succinate (ten to twenty times the concentration of malonate), the inhibition of succinic dehydrogenase by malonate, which is competitive, is overcome by the high concentration of succinate.

Much confirmatory evidence has more recently been supplied by experiments with isotopically labeled material. The distribution of labeled carbon was found to be in accordance with the assumption that the cycle is operative For example, the oxidation of C^{14}-labeled glucose or lactate, added to respiring tissues *in vitro*, leads to the formation of radioactive citrate (*15, 16*). Isotopes have been particularly helpful in studies of special aspects of the cycle and in work on microorganisms and plants to which reference is made in later sections of this chapter.

To sum up the evidence, there are several independent sets of observations which support a cyclic mechanism of the type shown in Scheme 2, and no alternative hypothesis which can explain the facts is available.

B. The Occurrence of the Cycle in Animal Tissues Other than Muscle

The main experiments on which the concept of the tricarboxylic acid cycle is based were carried out on striated muscle tissue, chiefly pigeon breast muscle, and on pigeon liver. The crucial experiments have been repeated with many other animal tissues and they indicate that the cycle occurs in all respiring tissues of all animals from protozoa to mammals. The earlier literature has been reviewed in the first edition of this book. References to more recent work are to be found in the *Annual Reviews of Biochemistry*.

It is true that some tissues and cells at first appeared to give negative results but these were later found to be due to special complications such as permeability barriers, or destruction of enzyme systems as the result of manipulating the tissues These experimental difficulties have been overcome by improved methods of handling the enzyme systems responsible for the reactions of the cycle.

C. Discussion of Special Aspects

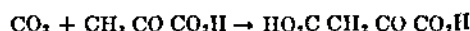
1 *Sequence in Which the Tricarboxylic Acids Arise*

It is obvious that the original cycle, as formulated in Scheme 2, is no more than a skeleton scheme, with numerous details to be filled in Until 1948 information on the mechanisms whereby the tricarboxylic acids arise from oxalacetate and pyruvate was very scanty. Though it was accepted as certain that the tricarboxylic acids are formed from these precursors, little was known about the intermediary stages of the process, and there was also some uncertainty regarding the sequence in which the three tricarboxylic acids arise Progress in this field was largely due to some theoretical considerations put forward by Ogston (*17*) and to enzyme studies of Stern and Ochoa (*18*)

The three tricarboxylic acids, citrate, *cis*-aconitate, and isocitrate, are always found together when they are formed in animal tissue, and it was therefore difficult to decide in which order they are formed For reasons which cannot be discussed in full (*19*) the order citrate → *cis*-aconitate → isocitrate was proposed originally But it was always clear (*20*) that this order was somewhat arbitrary

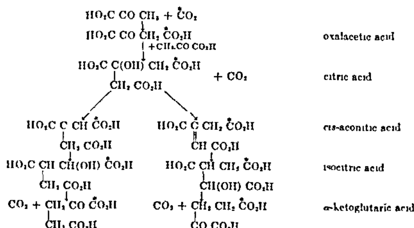
Evidence bearing on this question became available in 1941 from isotope experiments on pigeon liver by Wood *et al* (*21*), and by Evans and Slotin (*22*) The metabolism of this tissue is in many ways similar to that of pigeon muscle, as far as the oxidation of pyruvic acid is concerned, except that liver is capable of performing at least one additional reaction, the synthesis of oxalacetate from pyruvate and carbon dioxide (*21, 22*)

$$CO_2 + CH_3\,CO\,CO_2H \rightarrow HO_2C\,CH_2\,CO\,CO_2H$$



vented by malonate. If isotopic carbon dioxide is supplied, the metabolic fate of oxalacetate may be followed The reactions expected on the basis of the original theory are shown in Scheme 3 The carbon atom introduced

SCHEME 3
THE FATE OF CARBON DIOXIDE INTRODUCED BY CARBOXYLATION OF PYRUVIC ACID (BASED ON THE ORIGINAL CITRIC ACID CYCLE)*



* The carbon atom introduced in the form of CO_2 is marked by an asterisk. Owing to the symmetric structure of citric acid, two different modes of dehydration lead from citric acid to *cis*-aconitic acid

in the form of carbon dioxide is marked with an asterisk in the formulae, and it will be seen that, owing to the symmetric configuration of citric acid, the assimilated carbon is expected to appear in either of two carboxyl groups of aconitic, isocitric, and α-ketoglutaric acids. The experiments consisted of adding the isotope in the form of bicarbonate, isolating α-ketoglutaric acid as the 2,4-dinitrophenylhydrazone, and locating the isotopic carbon in the molecule of α-ketoglutaric acid The result of the experiments, i.e., the distribution of the isotopic carbon, was contrary to expectation in that the isotope was detectable only in the carboxyl group adjacent to the α-ketonic group (the "α-carboxyl"). This was taken to prove that Scheme 3 was incorrect (*21*, *22*). If the scheme were correct, it was thought, the α- and γ-carboxyl groups of α-ketoglutarate should contain equal amounts of the isotope, because the probability of the isotope appearing either in the α- or in the γ-carboxyl would be equal

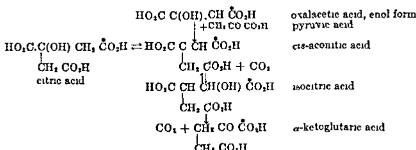
Wood *et al* (*21*) pointed out that a minor modification, already contemplated from the start as one of several possibilities, would meet the facts The main point of the modification was the assumption that the condensation of oxalacetate with pyruvate or a pyruvate derivative yields primarily *cis*-aconitate which is directly converted into isocitrate, whereas the formation of citrate is due to a side reaction (Scheme 4) If the rate of the side reaction between citrate and *cis*-aconitate is slow compared with the rates of the other reactions, it is to be expected that the fixed carbon appears predominantly in the carboxyl group of α-ketoglutaric acid adjacent to the keto group, as is actually the case.

A premise upon which this modification rested was the assumption that *the formation of citrate from cis-aconitate* is slow in comparison with the formation of isocitrate. It was thought earlier that this premise had an experimental basis (*23*), but in fact the rates of the two reactions catalyzed by aconitase are of the same order (*24*) The difficulty of explaining the asymmetric distribution of the isotopic carbon was removed in 1949 by Ogston (*17*, *25*), who showed that there is a fallacy in the above interpretation of the isotope experiments and that, in fact, citric acid does not necessarily behave as a symmetric molecule when combined with an enzyme. In view of the general importance of this concept it is more fully discussed in the section below

If the argument which was believed to rule out citrate as an intermediate has been found to be fallacious, it does not necessarily follow that the conclusion drawn from this argument was erroneous However, experiments of Stern and Ochoa (*18*), Ochoa *et al* (*26*), and Stern *et al* (*27*) showed conclusively that citrate and not aconitate is the primary product when oxalacetate and a second substance react to form a tricarboxylic acid These authors found that pigeon liver preparations

SCHEME 4

MODIFIED SCHEME SHOWING THE PRODUCTION OF α-KETOGLUTARIC ACID FROM OXALACETIC AND PYRUVIC ACIDS (21)*



* Isotopic C marked with asterisk. All the isotope appears in the α-carboxyl of α-ketoglutaric acid if the side reaction between citric and *cis*-aconitic acids is neglected The side reaction, as shown in Scheme 3, would produce a second isotopic form of *cis*-aconitic acid but its quantities would be small if the side reaction is slow

which have been virtually freed from aconitase form citrate on addition of oxalacetate plus acetate, or oxalacetate plus acetoacetate. It follows that *cis*-aconitate cannot be the primary condensation product

Furthermore, direct proof that citric acid, as postulated, reacts asymmetrically in the presence of enzymes was supplied by isotope experiments of Potter and Heidelberger (*28*), Wilcox *et al.* (*29*), Lorber *et al* (*30*), and by Martius and Schorre (*31*)

2 *The Asymmetric Behavior of Citric Acid and Other Compounds*

It was pointed out by Ogston (*17*) (in connection with the interpretation of isotope experiments on the formation of citric acid) that an enzyme which attacks a symmetric substrate may, under certain conditions, distinguish between "identical" groups of the substrate In other words, a symmetric molecule, when combined with an enzyme, may react asymmetrically. Whether this can occur depends on structural characteristics of both the enzyme and the substrate. The requirements are as follows·

1 The substrate molecule must be attached to the enzyme in a specific orientation The necessary degree of orientation is achieved if an attachment between substrate and enzyme occurred at not less than three points

2 The reactivities of the three sites where the combination occurs, must be different

3 The active site of the enzyme must be asymmetric (i.e , it must not possess a plane of symmetry).

4 The compound may have two, but not more, identical groups on the carbon atom affected by the enzyme reaction and the other two groups must differ from each other (i e , the compound must not possess more than one plane of symmetry) This point is covered in a more general form by the statement that the symmetric molecule must not possess a twofold or greater axis of symmetry (*32*). (An object possesses an *n*-fold axis of symmetry if rotation round the axis by $(360/n)°$ results in an arrangement with exactly the same appearance as the original) A full

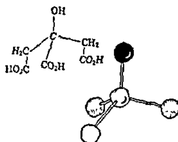


Fig 1 Diagram showing the spatial configuration of the tertiary carbon atom of the citric acid molecule (*23*)

and critical discussion of this problem has been given by *Hirschmann* (*33*) [see also refs (*34–36*)].

To appreciate Ogston's argument it is necessary to visualize the molecule concerned in space With citric acid as an example the treatment can be simplified by confining it to the tertiary carbon atom only, as is done in Fig 1. The tertiary carbon is pictured as being in the center of a tetrahedron, the attachment of the two $-CH_2\ COOH$ groups and the —COOH group being in a plane below, and that of the —OH in another plane above the tertiary carbon There is only one way in which citric acid can be connected with the enzyme if a three-point combination occurs, as shown by Fig. 2. If the two "dotted" points of attachment (Fig 2), which share the same shading because they combine with identical groups of the substrate, exhibit different geometric shapes or are catalytically different, the enzyme can obviously distinguish between the two symmetric $-CH_2.COOH$ groups This is provided that the substrate molecule can attach itself to only one side of the active site, as is the case with the surfaces of enzymes, in other words that it cannot attach itself from underneath in Fig 2

A difference in the catalytic properties of the three sites of combination is by no means an improbable event If, for the sake of argument, it be assumed that the attachment of the citric acid molecule to the enzyme is effected by the combination of the carboxyl groups of the substrate with

nitrogen atoms of the enzyme, catalytic differences would be expected if the nitrogen atoms belong to different amino acids

In the upper example of Fig. 2 it is arbitrarily assumed that the combination between enzyme and substrate is through the three carboxyl groups, but the argument is independent of any assumption on the nature of the groupings which enter the three-point combination. For example, a combination as pictured in the lower example has the same result in

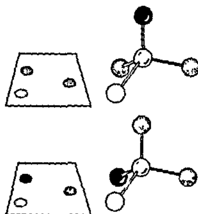


Fig 2 Illustration of the three-point attachment between citric acid and enzyme. The areas on the left-hand side of the diagram represent the enzyme surface It is assumed that combination between enzyme and substrate can occur where the patterns of enzyme model and substrate model match There is only one position in which the citric acid molecule can be placed on the enzyme if a three-point attachment takes place (23)

that it makes a symmetric material asymmetric, an essential aspect is the fact that only one kind of asymmetry can be produced.

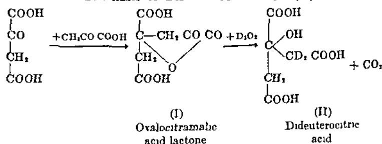
If the labeling of α-ketoglutarate observed experimentally is to be accounted for, and if citrate is to be included as an intermediate in the cycle, it is thus necessary to postulate that a "three-point attachment" is operative when citrate is converted to isocitrate It is further necessary to postulate that a three-point attachment is operative when citrate is formed from oxalacetate and acetyl coenzyme A For example, oxalacetate might combine at two points and acetyl coenzyme A at a third.

Isotope experiments of Potter and Heidelberger (28), Wilcox *et al* (29), Lorber *et al* (30), and by Martius and Schorre (31) demonstrate directly the asymmetric behavior of citric acid in the presence of enzymes. Potter and Heidelberger (28) isolated radioactive citric acid from a rat liver homogenate which had been incubated with $C^{14}O_2$ The labeled

citrate, which had presumably arisen by the mechanism shown in Scheme 3, was then added to another rat liver homogenate which, in the presence of arsenite, converted the citrate into α-ketoglutarate The radioactivity of the isolated α-ketoglutarate was found to be located entirely in the α-carboxyl group Similar experiments giving the same result were carried out by Lorber *et al.* (*30*). They proved the asymmetric behavior of the citric acid molecule and that only one of the pathways in Scheme 3, viz, the left-hand one, was operative Additional evidence was provided by experiments of Wilcox *et al.* (*29*) Asymmetric sodium citrate containing C^{14} was prepared by the reaction of $NaC^{14}N$ with levorotatory γ-chloro-β-carboxy-β-hydroxy butyric acid. The labeled citrate was added to rat liver homogenate containing 0.003 *M* arsenite. The 2,4-dinitrophenyl hydrazone of α-ketoglutaric acid was isolated and was found to carry the isotope entirely in the γ-carboxyl group

Martius and Schorre (*31*) prepared dideuterocitric acid starting from pyruvic and oxalacetic acids (Scheme 5) By aldol condensation the two

SCHEME 5
SYNTHESIS OF DIDEUTEROCITRIC ACID (*31*)



ketonic acids yield oxalocitramalic acid lactone (I). The latter can be resolved through the acid brucine salt into the two stereoisomers Treatment of the lactone with D_2O_2 in D_2O yields (II) The L- and D-forms yield—a remarkable observation—optically active substances giving a specific rotation in water of about 1°, which on addition of ammonium molybdate rises to 33°. The two stereoisomers of (II) were added to pigeon breast muscle in the presence of arsenite and O_2, and the α-ketoglutarate arising by oxidation was isolated as the dinitrophenyl hydrazone The levorotatory acid yielded an α-ketoglutarate containing all the deuterium of the starting material, but the dextrorotatory form had lost all deuterium in the reaction, and the racemate had lost exactly half These results constitute conclusive evidence of the asymmetric breakdown of citric acid, as postulated by Ogston

The question may be asked whether a three-point combination is a

likely event in biological material Ogston has given a good reason for believing that this is the case. He has pointed out that substances other than citric acid can react "asymmetrically" in the presence of enzymes, for instance, when an optically active substance arises from an inactive precursor Examples of such reactions are the formation of L-malate from fumarate, or of L-isocitrate from *cis*-aconitate, or of L-lactate from pyruvate In these cases, too, a three-point attachment can explain the action of the enzymes This may be elaborated for the case of fumarase.

In Fig 3 the spheres indicate the spatial arrangement of the four carbon atoms of fumaric acid. These lie in one plane, the two terminal

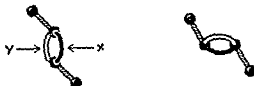


FIG 3 Diagram illustrating the possible role of a three-point combination between enzyme and substrate in the formation of optically active malic acid from fumaric acid (see text) (25)

carbon atoms being in the *trans*-position If we assume that fumaric acid combines with the enzyme at two points, say, the carboxyl groups, then the fumarate molecule can assume two different positions on the surface of the enzyme molecule (because the single bonds connecting the terminal carbon atoms with the α and β carbon atoms are not rigid but can rotate). The two positions are shown in Fig 3, and the point to note is that the position of the two bonds between the α and β carbon atoms differs in relation to the enzyme surface in the two possible arrangements. In one case the double-bond drawn in solid black (x), in the other that drawn in outline (y), comes to rest on the surface of the enzyme This difference in space is very likely to affect the reactivity of the two components of the double bond, one being likely to react in preference to the other Now, if the elements of water are added to fumarate to saturate the double bonds, then optically active malic acid is formed if H and OH replace selectively the valencies of either bond x or bond y DL-Malate is formed if bonds x and y have *an equal chance of reacting*. Hence, any arrangement in space which will cause a difference in the reactivity of the two components of the double bond will produce optically active compounds. A three-point combination of fumaric acid with the enzyme could result in a differential spatial arrangement, whereas a two-point combination of fumarate would allow two positions in space.

The third attachment would fix the molecule rigidly in one position and would cause a differentiation in the reactivity of the double bond

One further point remains to be amplified. The elements of water can be added to each double bond of fumarate in two ways, which differ with regard to the places of the OH and H groups. However, as Fig. 4 makes clear, the manner in which the elements of water are added does not affect the final result, provided that OH and H are added to the same bond. In the diagram the short line indicates the bond holding the OH group It extends into space above or below the diagram. In the left-

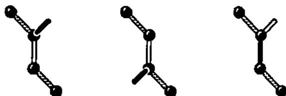
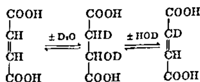


FIG 4 Illustration of the steric configuration of malic acids arising by hydration of fumaric acid

hand figure the hydroxyl is attached to the β carbon and in the center figure to the α carbon of bond x By rotating either of the figures through 180°, they become identical In the right-hand figure the hydroxyl is attached to bond y, and it cannot be made identical with the other figures by rotation It is therefore different from them. The inference is that in order to yield optically active malic acid the fumarate molecule must be so directed that one double-bond component only can react, but no direction needs to be exerted on the manner in which the elements of water are attached *(25)*.

That fumarase behaves asymmetrically (or "stereospecifically") has also been demonstrated by exposing fumarate to fumarase in the presence of deuterium oxide *(37–39)*. If fumarate reacted symmetrically on the β carbon it would lead to the incorporation of deuterium into fumarate, because the back reaction from malate could involve either H or D.



In fact, the reaction on the right does *not* occur, and no deuterofumarate is formed on prolonged incubation. Thus the fumarate reaction is asymmetric not only with respect to the carbon atom to which the hydroxyl

is added, but also with respect to the carbon to which the hydrogen is added. An analogous situation applies to aconitase (*40*), and to aspartase (*41*) which catalyzes the conversion of fumarate and ammonia to L-aspartic acid

Examples of other symmetric molecules which behave asymmetrically in the presence of enzymes are glycerol (*42*, *43*) and ethanol (*44*)

3. *The Enzymes of the Tricarboxylic Acid Cycle*

Each stage of the cycle requires a specific enzyme or enzyme system Although it is possible to separate the actions of the different enzymes, the isolation of the individual enzymes completely free of other enzyme activities has proved difficult So far only two enzymes, fumarase (*45*) and the condensing enzyme (*46*, *47*) have been obtained in a crystalline form and even the four times recrystallized condensing enzyme still contains small amounts of isocitric dehydrogenase (*48*)

a CONDENSING ENZYME The reversible reaction catalyzed by the condensing enzyme which leads to the formation of citrate from acetyl coenzyme A and oxalacetate, may be formulated as follows



$$\text{Acetyl-S CoA} + \text{oxalacetate}^{2-} + H_2O \rightleftharpoons \text{citrate}^{3-} + \text{HS CoA} + H^+$$

The equilibrium of the reaction is very much in favor of the formation of citrate Citroyl coenzyme A may be expected to be an intermediate but its formation has not been demonstrated It would presumably exist only as an enzyme-bound intermediate

b. ACONITASE Aconitase catalyzes the reversible interconversion of the three tricarboxylic acids



$$\text{Citrate} \rightleftharpoons \textit{cis}\text{-aconitate} \rightleftharpoons \text{isocitrate}$$

At 38° and pH 6 8 the equilibrium mixture contains 89.1% citrate, 4 3% isocitrate, and 6 6% *cis*-aconitate (*49*) At 25° and pH 7.4 the corresponding figures are 90 9% citrate, 6.2% isocitrate, 2 9% *cis*-aconitate (*50*)

However, doubt as to whether the intermediary formation of *cis*-aconitate is obligatory was expressed by Martius and Lynen (*51*) and Friedrich-Freksa and Martius (*52*) who failed to observe a lag period when measuring the kinetics of the conversion of citrate into isocitrate. If the conversion were represented by the above scheme a lag period would be expected during which a maximum concentration of *cis*-aconitate is built up. Using different experimental conditions, Krebs and Holzach (*53*), and Morrison (*54*) in fact observed the expected lag

period In the early stages of incubation of the enzyme with citrate as the substrate *cis*-aconitate accumulated but little isocitrate was formed. As expected the rate of isocitrate formation increased with time

More recently, Speyer and Dickman (*55*) investigated the reaction by comparing the incorporation of deuterium from D_2O into citrate, starting with isocitrate or *cis*-aconitate, and reached the conclusion that *cis*-aconitate is not an obligatory intermediate.

The citrate formed from isocitrate contained much less deuterium than the citrate formed from *cis*-aconitate. Speyer and Dickman (*55*) explained this by the assumption that the interconversion between citrate and isocitrate can occur by an intramolecular rearrangement not involving *cis*-aconitate The rearrangement is postulated to proceed via an intermediate common to all three tricarboxylic acids Speyer and Dickman formulate this intermediate as a tricarboxylic acid carbonium ion complexed to aconitase, cysteine, and Fe^{++}. The inclusion of the latter two substances is based on the observations that solutions of pure and partially purified aconitase are stabilized and activated by the addition of Fe^{2+} and cysteine (*54, 56, 57*) The enzyme-bound intermediate is assumed to rearrange readily and reversibly to yield enzyme-bound citrate, isocitrate, or *cis*-aconitate. If this view proves correct *cis*-aconitate should be considered as a side product rather than an obligatory intermediate.

Although aconitase is a highly specific enzyme acting only upon the three substances of the aconitase system, viz , citrate, isocitrate, and *cis*-aconitate, it has the unusual property of catalyzing two different reactions· the dehydration of citric acid involves a hydroxyl attached to a tertiary carbon; the dehydration of isocitrate involves a hydroxyl attached to a secondary carbon No other substances are known to be attacked by the enzyme. Earlier workers (*58, 59*) assumed that two different enzymes, α and β aconitase, were responsible for the dehydration of citrate and isocitrate However, attempts to resolve the enzyme from pig heart have failed, and on purification the relative activities of α and β aconitase seem to remain constant (*60, 61*).

Ogston (*25*) has put forward an idea which removes the difficulty of ascribing both reactions to one enzyme. It is a development of his theory of a "*three-point*" *combination between enzyme and substrate* As already explained, the fumarate molecule in order to yield optically active malic acid must be so placed on the enzyme surface that only one double-bond component can react, but no direction need be exerted on the manner H and OH are distributed If it is now assumed that aconitase is constructed analogously to fumarase in that again no direction is exerted on the elements of water when they combine with aconitic acid,

it is seen at once that two different compounds arise, namely, citric and isocitric acids. The occurrence of the reverse reaction would follow from the requirement of catalytic reversibility.

On the other hand, experiments by Neilson (*62*) suggest that the aconitase of *Aspergillus niger* may consist of two enzymes [see also Racker (*63*)].

c ISOCITRIC DEHYDROGENASE (TPN specific) There are two enzyme systems which catalyze the series of reactions leading from *d*-isocitrate[1] to α-ketoglutarate One is TPN specific, the other DPN specific. Both require Mg^{2+} or Mn^{2+} and are widely distributed, but the activity of the former is generally much greater than that of the latter The reaction catalyzed by the TPN-specific enzyme (*64–66*) is reversible and is described by the following reaction scheme



$$d\text{-Isocitrate}^{3-} + \text{TPN}^+ \xrightleftharpoons{Mn^{2+} \text{ or } Mg^{2+}} \alpha\text{-ketoglutarate}^{2-} + CO_2 + \text{TPNH} + H^+$$

This involves at least two major steps The first is dehydrogenation which is expected to lead to oxalosuccinate



$$d\text{-Isocitrate}^{3-} + \text{TPN}^+ \rightleftharpoons \text{oxalosuccinate}^{3-} + \text{TPNH} + H^+$$

The second is a decarboxylation of oxalosuccinate.



$$\text{Oxalosuccinate}^{3-} + H^+ \xrightleftharpoons{Mn^{2+} \text{ or } Mg^{2+}} \alpha\text{-ketoglutarate} + CO_2$$

The first stage requires pyridine nucleotide but no divalent cation (though it is slower in its absence), while the second requires Mn^{2+} or Mg^{2+} but no pyridine nucleotide In the absence of divalent metal oxalosuccinate is the end product when a purified enzyme preparation is used All efforts to separate the two reactions by purification have failed (*67–70*) The ratio of the two activities remained constant during purification and these observations have led to the conclusion that the dehydrogenase and decarboxylase activities are associated with a single protein The situation is analogous to that found in the case of the "malic enzyme" This enzyme catalyzes the formation of pyruvate and CO_2 from malate without the intermediary formation of free oxalacetate Moyle and Dixon (67) have therefore attributed the combined isocitric dehydrogenase and oxalosuccinate decarboxylase activity to one enzyme

[1] *d*-Isocitrate designates the naturally occurring dextrorotatory form Since isocitrate has two asymmetric centers it occurs in four forms In the natural form the carbon carrying the hydroxyl group probably has the same configuration as the α-carbon of L-serine and this form is therefore also referred to as $L_s(+)$ isocitric acid (see H B Vickery, *Science* **113**, 314, 1951, H B Vickery and D G Wilson, *J Biol Chem* **233**, 14, 1958, M Winitz, S M Birnbaum, and J P. Greenstein, *J Am. Chem Soc* **77**, 716, 1955)

system for which they propose the name "isocitric enzyme." Isociti oxalosuccinate, and α-ketoglutarate are taken to occupy the same ac site on the enzyme and the dissociation of the enzyme-oxalosucci complex is assumed to be negligible compared to the dissociation of other two substrate enzyme complexes (*71, 72*). In other words, enzy bound oxalosuccinate rather than free oxalosuccinate is assumed to the intermediate

The TPN-specific isocitric dehydrogenases from pig heart (*67*, yeast (*71*), *Aspergillus niger* (*72*), and *Trypanosoma cruzi* (*73*) h similar properties

d Isocitric Dehydrogenase (DPN specific). The DPN-spe isocitric dehydrogenase has been separated from the TPN-spe enzyme in several materials, such as heart muscle, pigeon breast mus and bakers' yeast (*71, 72*) This separation indicates that there are entirely different enzymes rather than one enzyme reacting with b DPN and TPN The DPN-specific enzymes also require Mg^{2+} or Mi but differ from the TPN-specific enzymes in several ways The ye enzyme (*71*) requires the presence of catalytic quantities of adenosine phosphate (the adenosine 2'- and 3'-phosphates, adenosine or ATP ineffective) Moreover, the reversibility of the reaction, i e. the reduct of oxalosuccinate and the reductive carboxylation of α-ketoglutar could not be demonstrated with the DPN specific yeast or heart enzy (*71*) The DPN enzyme from animal tissues is not activated by adenosi 5'-phosphate

The TPN-specific enzyme occurs mainly in the soluble fraction the cytoplasm. Thus, in rat liver about 75–90% are located in the cy plasm and about 10–25% in mitochondria (*74*). The DPN-speci enzyme of heart, muscle, and kidney occurs exclusively in mitochondr It is absent or virtually so from many other tissues.

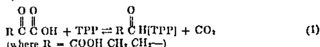
According to Kornberg and Pricer (*71*) the purified DPN- enzyme yeast does not decarboxylate oxalosuccinate which suggests that oxalosu cinate may not be an intermediate As in the case of the TPN-specific d hydrogenase, the term "isocitric enzyme" may therefore be appropria

The reason for the existence of two different enzyme systems with one cell catalyzing the same reaction is not yet fully understood

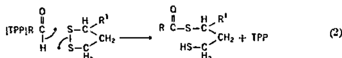
e α-Ketoglutaric Dehydrogenase System The conversion α-ketoglutarate to succinate requires the participation of diphosph thiamine (TPP; cocarboxylase), α-lipoic acid (dextrorotatory 6,8-thioct acid), coenzyme A, diphosphopyridine nucleotide and magnesium ion and involves a series of separate steps which have been tentative formulated as follows (*75–78*)

The first step is taken to be a reaction between α-ketoglutarate an

TPP in which a succinic semialdehyde-TPP complex is formed and CO_2 is liberated (*79, 80*)

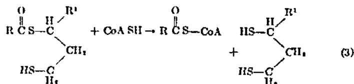


Evidence in support of this reaction is the observation that TPP catalyzes the exchange of $C^{14}O_2$ with α-ketoglutarate in preparations of "α-ketoglutaric oxidase" of pig heart (*80*). According to Breslow (*81*) and to Krampitz *et al.* (*82*) the aldehyde is probably attached to the 2-position of the thiazole ring of thiamine. The succinic semialdehyde-TPP complex is assumed to react with the disulfide form of α-lipoic acid in such a manner that the aldehyde group of succinic semialdehyde is oxidized to the corresponding carboxyl and the disulfide reduced to the dimercaptan, with concomitant formation of an acylthiol

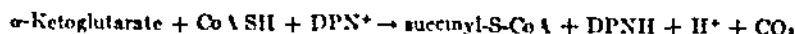


TPP is regenerated in this reaction. Evidence for the "succinylation" of lipoic acid, has been provided by experiments in which C^{14}-labeled *α-ketoglutarate was incubated with relatively large amounts of purified* enzyme and hydroxylamine in the absence of coenzyme A. Substantial amounts of radioactive succinhydroxamic acid were formed and identified by isolation (*78*). This is interpreted as a demonstration of the formation of a succinyl enzyme. Further experiments by Sanadi *et al.* (*78*) indicated that the active site of the enzyme to which the succinyl group is attached is bound α-lipoic acid.

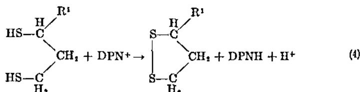
In the next step the succinyl group is transferred from α-lipoic acid to the thiol group of coenzyme A, forming reduced α-lipoic acid and succinyl coenzyme A



This "transacylation" or "trans-succinylation" is indicated by the coenzyme A requirement of the α-ketoglutarate oxidase system and the actual formation of succinyl coenzyme A by the over-all reaction:



which represents the sum of (1), (2), (3), and (4). Furthermore the analogous transfer of the acetyl group between reduced lipoic acid and coenzyme A has been found to be catalyzed by preparations of the pyruvic oxidase systems of *Escherichia coli* (*83*). In order to be able to react again in the oxidation of another molecule of the aldehyde-TPP complex the reduced lipoic acid has to be reoxidized to the disulfide This is accomplished by coupled reduction of DPN under the influence of lipoic acid dehydrogenase (*84–86*):

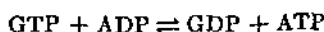


The enzyme catalyzing this reaction, lipoic acid dehydrogenase, has been shown to be a component of α-ketonic acid oxidase systems (*84–86*) The succinyl coenzyme A formed by (3) reacts with guanosine diphosphate (GDP) and orthophosphate (P) to regenerate coenzyme A and to form guanosine triphosphate (GTP) and succinate (*87*, *88*).

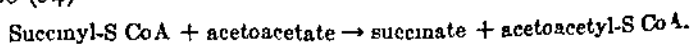


The reaction is a composite one as follows from the number of reactants, and also from the fact that succinyl coenzyme A can be split to succinate and coenzyme A in the presence of arsenate but in the absence of GDP (*89*). Moreover oxygen from O^{18}-labeled phosphate appears in one of the carboxyl groups of the succinate formed (*90*) A phosphorylated succinate derivative or a phosphorylated coenzyme A may therefore be intermediates in this reaction, though neither has yet been isolated or identified. Chemically prepared succinyl phosphate does not replace succinyl coenzyme A (*91*, *92*)

The GTP formed by reaction (5) interacts with ADP to form GDP and ATP (*87*) ADP can be replaced by IDP The reaction is catalyzed by nucleoside diphosphokinase:



Succinyl-coenzyme A, apart from reacting according to (5), can be hydrolyzed under the influence of deacylase (or *thioesterase*) to succinate and coenzyme A (*93*) or it can be deacetylated by transfer reactions of the type (*94*)



Lipoic acid probably always reacts in this system in a protein-bound form (*77*, *78*, *95*, *96*), although free succinyl lipoic acid may arise by

secondary transfer from succinyl coenzyme A to reduced lipoic acid if transacylase is present (*78*).

f SUCCINIC DEHYDROGENASE Succinic dehydrogenase transfers two hydrogen atoms from succinate to ferricytochrome *b* (*97*):



$$\text{Succinate} + 2\ \text{cyt}\ b\text{-}Fe^{3+} \rightarrow \text{fumarate} + 2\ \text{cyt}\ b\text{-}Fe^{2+} + 2\ H^+$$

The enzyme is tightly bound to the structure of the mitochondria, but can be solubilized by drying mitochondria with acetone and extracting the powder with neutral buffers after blending (*98*) The purified enzyme from beef heart and yeast has been found to contain four atoms of nonhemin iron and one molecule of flavin per molecule of protein (*98*) The analogous enzyme from *Micrococcus lactilyticus* has a much higher iron content (*99*) The purified succinic dehydrogenases isolated from animal tissues, yeast, and *Micrococcus lactilyticus* also catalyze the reduction of fumarate to succinate, reduced dyes of low potential, such as leuco diethylsafranin, serving as hydrogen donors There is no evidence that "fumaric reductase" is a separate enzyme (*100, 101*)

g FUMARASE. The stereospecificity of the addition of water to fumarate has already been discussed (Section II,C,2) The reversible interconversion of L-malate and fumarate leads to an equilibrium when the solution contains 18 45% fumarate and 81 55 L-malate (pH 7 4; 25°) (*102*). Lowering the temperature shifts the equilibrium in favor of L-malate (*103*) Other characteristics of this enzyme have been summarized by Massey (*104*)

h MALIC DEHYDROGENASE. The equilibrium of the reversible reaction



$$\text{L-Malate}^{2-} + DPN^+ \rightleftharpoons \text{oxalacetate}^{2-} + DPNH + H^+$$

catalyzed by malic dehydrogenase favors the reaction's going from left to right when the concentrations of the reactants are of the same order of magnitude However, the physiological concentration of oxalacetate is extremely low (*105*), and the reaction proceeds readily from left to right under physiological conditions The enzyme also catalyzes the dehydrogenation of other α-hydroxydicarboxylic acids (*106*).

4 *Diagram of the Tricarboxylic Acid Cycle*

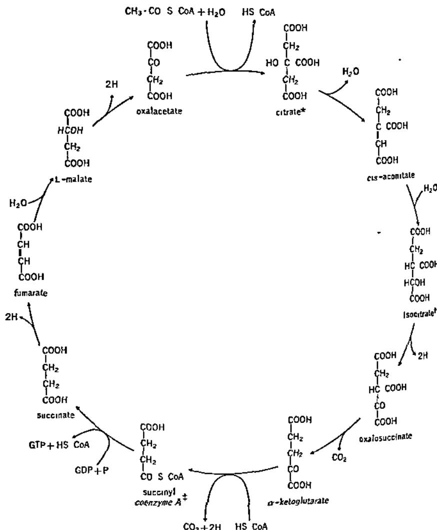
Diagram A summarizes the present knowledge of the intermediary stages of the cycle

5 *Intracellular Location of the Enzymes of the Tricarboxylic Acid Cycle*

The chief intracellular sites of the tricarboxylic acid cycle are the mitochondria (*107*). This is borne out by the fact that the isolated mito-

Diagram A. The Tricarboxylic Acid Cycle

The diagram illustrates the complete combustion of one acetic acid equivalent *The end products shown are CO_2, water, and 4 pairs of hydrogen atoms* The latter subsequently react through the electron carrier chain with molecular oxygen to form water A special point to be noted is the fact that the β-carboxyl of oxalacetate becomes the α-carboxyl of α-ketoglutarate and that both carbon atoms of the acetyl group entering the cycle remain in the C_4 intermediates during the first turn of the cycle. Reversibility of reactions is not indicated



* Citrate can yield isocitrate without *cis*-aconitate occurring as a free intermediate *(55)*

† Isocitrate can yield α-ketoglutarate without oxalosuccinate occurring as a *free* intermediate *(68)*

‡ Succinyl coenzyme A can also yield succinate by the succinyl coenzyme A deacylase *(93)* and succinyl coenzyme A transferase reactions *(94a)*.

chondria, after separation from other cell organelles, oxidize substrates via the cycle at a high rate (*108*), and that, with one major exception, the dehydrogenases of the cycle are predominantly located in the mitochondria (*109–111*) The exception is isocitric dehydrogenase which in many tissues also occurs in the soluble fraction of the cytoplasm: in liver homogenates about 80–90% of the TPN-specific isocitric dehydrogenase is in the soluble fraction (*112, 74, 113*) However, in addition to some of the TPN enzyme, mitochondria contain the DPN specific isocitric dehydrogenase (*114*) and the fact that mitochondria can oxidize substrates to completeness indicates that the intramitochondrial isocitric dehydrogenases are sufficiently active to cope with the citrate arising during the cycle at high oxidation rates

The physiological role of the TPN-specific isocitric dehydrogenase in the soluble fraction is uncertain

Osawa *et al.* (*115*) have reported that oxidative phosphorylation may occur in isolated nuclei of calf thymus without participation of cytoplasmic components This implies that oxidative reactions can also occur in the nuclei While the cell fraction containing the nuclei has long been known to absorb O_2 (though at a low rate) it had previously been assumed that this was due to contamination with mitochondria. The experiments of Osawa *et al* suggest that nuclear respiration and phosphorylation play a specific role in the synthesis of protein within the nuclei

6 *Observations on the Intact Animal*

It is in general not easy to prove the occurrence of intermediary processes *in vivo* in higher animals because the complexity of biological systems limits the possibilities of quantitative experiments Such evidence as is available from *in vivo* experiments is all consistent with the operation of the cycle Thus the injection of malonate leads to the accumulation of succinate in the urine (*116*) and in a variety of tissues (*117*) Injection of fluoroacetate (*118, 119*) leads to the accumulation of citrate in many tissues After the injection of compounds labeled with C^{14}, the distribution of the isotope in cell constituents is found to be that which can be predicted by assuming that the cycle is operative (*120–123*)

III. The Cycle in Microorganisms

A Introductory Remarks

Until about 1948 the pathway of oxidations in microorganisms was largely obscure Although a large number of oxidative reactions had been demonstrated—Quastel (*124*) recorded over 50 different dehydro-

genation reactions in *Escherichia coli* and den Dooren de Jong (*125*) found 79 substances which can serve as the sole source of carbon for a strain of pseudomonads—it had not been possible to link these reactions together in a coherent series representing the pathways of oxidation of the nutrients The type of experiment which had provided evidence on the nature of intermediary stages in animal tissue—the demonstration of the oxidizability of certain substrates and of the catalytic action of di- and tricarboxylic acids, the use of specific inhibitors such as malonate and the use of isotopes—gave in many cases inconclusive information when applied to microorganisms It was appreciated at an early stage that permeability barriers present special difficulties in the study of microorganisms where such barriers are more marked than in animal tissues More recent work has fully confirmed the importance of permeability barriers for the behavior of metabolites and inhibitors.

The newer development began in 1942 when Lynen (*126*) reported experiments on yeast cells which suggested the occurrence of the tricarboxylic acid cycle in yeast preparations, but the evidence was not generally accepted as conclusive because it fell short in satisfying the quantitative requirements. From 1948 onward, however, new observations, especially those based on the use of isotopic tracers have demonstrated conclusively in numerous cases that the cycle represents the main *terminal pathway* of acetate oxidation in microorganisms This may be said to be true for every aerobic organism which has been thoroughly examined Certain photosynthetic bacteria are possibly exceptions (*127, 128*).

B Observations on Yeast

All the steps of the cycle have been shown to occur in yeast· Citrate accumulates when magnesium or barium acetate (0.2 *M*) is added to yeast cells (*129–131*) or when acetate and oxalacetate are added to extracts from yeast cells (*132*). Added citrate can be converted to α-ketoglutarate by various types of yeast preparations (*71, 133, 134*) though not by intact cells Succinate can be oxidized to fumarate (*135*), and malate to oxalacetate (*135*), and fumarase (*134, 136*) and aconitase (*131–134*) have been shown to be present Furthermore, isotope data with labeled acetate agree with the assumption that acetate is converted to citrate and succinate (*131, 137, 138*).

However, many of the above reactions appeared to be relatively slow, especially the oxidation of citrate and malate Thus on addition to a suspension of yeast cells these substances are not appreciably attacked (see Table II)

An obvious method of overcoming permeability barriers is to experi-

ment with disrupted cells, but all attempts to obtain cell-free yeast extracts capable of oxidizing acetate have so far been unsuccessful Yeast cells disintegrated by shaking with Ballotini beads oxidize ethanol to acetate and attack various di- and tricarboxylic acids of the cycle (*139–143*) but the oxidation is incomplete, and acetate is not attacked The rates of oxidations in yeast fragments generally, and in particular those of fumarate and malate are low.

Another way of removing permeability barriers is freezing and thawing, as Dixon and Atkins (*144*) and Lynen (*145*) have shown For example,

TABLE II
RESPIRATION OF BAKERS' YEAST IN THE PRESENCE OF VARIOUS SUBSTRATES

Substrate	Q_{O_2}
Glucose	−103
Ethanol	−112
Acetate	− 99
Pyruvate	− 49
Succinate	− 7 2
Citrate	− 7 1
Fumarate	− 6 0
None	− 7 8

malate and citrate which are not attacked by untreated yeast cells are readily converted into fumarate and isocitrate respectively by yeast cells which have been exposed to the temperature of solid CO_2 or liquid air (*140*) These reactions take place within the cells, the supernatant having no fumarase and aconitase activity (*146*) Suspensions of cold-treated cells oxidize acetate (though at a lower rate than normal cells) but fail to oxidize citrate or fumarate. Since these substances penetrate the cold-treated cells this observation was taken to suggest that the oxidation of acetate does not involve citrate or fumarate as intermediates, a conclusion supported by other observations (*146*) However, recent isotope work described below has shown this argument to be fallacious. The findings can be satisfactorily explained by the assumption that suspensions of cold-treated cells contain two types of cells, undamaged ones capable of oxidizing acetate and impermeable to citrate and fumarate, and damaged ones which have lost the capacity to oxidize acetate but are permeable to di- and tricarboxylic acids.

A technique which has proved adequate to deal with the problem has been introduced by Krampitz and his collaborators (*147–149*). It consists of the study of the labeling pattern of the *intracellular* inter-

mediates after addition of C^{14}-labeled acetate Using this technique, which requires relatively large amounts of cells, DeMoss and Swim (*150*) found that the isotope was located in citrate, α-ketoglutarate, succinate, fumarate, malate, and the four amino acids readily arising from intermediates of the tricarboxylic and glyoxylate cycles, viz, glutamate, aspartate, alanine, and glycine The di- and tricarboxylic acids were found to be in isotopic equilibrium with each other. No appreciable quantities of other labeled compounds appeared and the labeling pattern was as expected on the basis of the operation of the tricarboxylic acid and glyoxylate cycles

These results are borne out by experiments on the distribution of $C^{14}O_2$, fixed by respiring bakers' yeast (*151*) and especially by further observations (*152*) on the time course of the isotope distribution in bakers' yeast after addition of C^{14}-acetate, when this served as the sole source of carbon The intracellular intermediates of the cycle and compounds closely related to it (glutamate, aspartate, polysaccharide) became labeled within seconds. The rates of formation of the intracellular intermediates from acetate, as calculated from the concentrations of the metabolites, their specific activity and the specific activity of the added acetate, proved to be of the same order as the rates calculated from the measurement of the oxygen consumption of yeast The speed of the reactions of the cycle is thus sufficient to justify the conclusion that the cycle is the chief pathway of acetate oxidation

The oxygen consumption in these experiments (*151*) amounted to about 70% of the value required for the complete combustion of acetate, owing to the assimilation of some acetate A part of the assimilated acetate appeared in the form of citrate and malate, the intracellular concentration of which rapidly increased with time This means that a major part of the assimilated acetate reacted via the glyoxylate cycle

C. Observations on Bacteria

There are many observations recorded in the literature on the occurrence and rates of the component reactions of the cycle, and of the behavior of isotopic substances, which are in accordance with the assumption that the cycle is the main pathway of oxidation of acetate in many bacteria A list of organisms in which various aspects of the problem have been investigated is given in Table III.

Until recently data remained which were difficult to reconcile with the assumption that the cycle represents the *only* major pathway of acetate oxidation. The situation resembled that described above for yeast. Thus in *Escherichia coli*, citrate is not oxidized and cannot serve as an energy source for growth (*153–155*). Further, in this organism

added α-ketoglutarate remained unlabeled when isotopic acetate was oxidized while added succinate, malate, and fumarate became radioactive (*156*). The analysis of the labeling pattern of intracellular intermediates by Krampitz and his collaborators (*156–158*) has shown in several bacteria (as in the case of yeast) that these findings can be

TABLE III
BACTERIA IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE TRICARBOXYLIC ACID CYCLE HAS BEEN OBTAINED

Organism	References
Aerobacter aerogenes	Wong and Ajl (*153*), Gilvarg and Davis (*154*)
Acetobacter pasteurianum	King, *et al* (*155*)
Acetobacter peroxidans	Atkinson (*156*)
Alcaligenes faecalis	Tourtellotte *et al* (*157*)
Azotobacter vinelandii	Stone and Wilson (*158*)
Bacillus cereus	Beck and Lindstrom (*159*)
Bacillus subtilis	Wiame and Bourgeois (*160*)
Brucella abortus	Altenbern and Housewright (*161*)
Corynebacterium creatinovorans	Fukui and Vandemark (*162*)
Escherichia coli	Swim and Krampitz (*149*), Gilvarg and Davis (*154*)
Micrococcus lysodeikticus	Saz and Krampitz (*163*)
Mycobacterium phlei	Blakley (*164*)
Mycobacterium tuberculosis	Millman and Youmans (*165*)
Pasteurella pestis	Englesberg and Levy (*166*)
Propionibacterium pentosaceum	Delwiche and Carson (*167*)
Pseudomonas aeruginosa	Campbell and Stokes (*168*)
Pseudomonas fluorescens	Barrett and Kallio (*169*), Kogut and Podoski (*170*)
Rhodopseudomonas capsulatus	Stoppani *et al* (*171*)
Rhodospirillum palustris	Crook and Lindstrom (*172*)
Rhodospirillum rubrum	Crook and Lindstrom (*172*)
Staphylococcus aureus	Stedman and Kravitz (*173*)

accounted for by permeability barriers The intracellular metabolites become labeled on addition of C^{14}-acetate as expected, but some of the di- and tricarboxylic acids do not mix with the added metabolites in the medium This has been established, among others for *Micrococcus lysodeikticus* (*147*) and *Escherichia coli* (*148*).

The nature of the permeability barrier which separates internal and external metabolites, has recently been investigated by Campbell and [illegible]

of certain metabolites into the cell has been *shown to depend on the* presence, within the cell, of specific proteins which act as transporting

mediates after addition of C^{14}-labeled acetate Using this technique, which requires relatively large amounts of cells, DeMoss and Swim (*150*) found that the isotope was located in citrate, α-ketoglutarate, succinate, fumarate, malate, and the four amino acids readily arising from intermediates of the tricarboxylic and glyoxylate cycles, viz, glutamate, aspartate, alanine, and glycine. The di- and tricarboxylic acids were found to be in isotopic equilibrium with each other. No appreciable quantities of other labeled compounds appeared and the labeling pattern was as expected on the basis of the operation of the tricarboxylic acid and glyoxylate cycles

These results are borne out by experiments on the distribution of $C^{14}O_2$, fixed by respiring bakers' yeast (*151*) and especially by further observations (*152*) on the time course of the isotope distribution in bakers' yeast after addition of C^{14}-acetate, when this served as the sole source of carbon The intracellular intermediates of the cycle and compounds closely related to it (glutamate, aspartate, polysaccharide) became labeled within seconds The rates of formation of the intracellular intermediates from acetate, as calculated from the concentrations of the metabolites, their specific activity and the specific activity of the added acetate, proved to be of the same order as the rates calculated from the measurement of the oxygen consumption of yeast. The speed of the reactions of the cycle is thus sufficient to justify the conclusion that the cycle is the chief pathway of acetate oxidation.

The oxygen consumption in these experiments (*151*) amounted to about 70% of the value required for the complete combustion of acetate, owing to the assimilation of some acetate A part of the assimilated acetate appeared in the form of citrate and malate, the intracellular concentration of which rapidly increased with time This means that a major part of the assimilated acetate reacted via the glyoxylate cycle

C. Observations on Bacteria

There are many observations recorded in the literature on the occurrence and rates of the component reactions of the cycle, and of the behavior of isotopic substances, which are in accordance with the assumption that the cycle is the main pathway of oxidation of acetate in many bacteria A list of organisms in which various aspects of the problem have been investigated is given in Table III

Until recently data remained which were difficult to reconcile with the assumption that the cycle represents the *only* major pathway of acetate oxidation The situation resembled that described above for yeast. Thus in *Escherichia coli*, citrate is not oxidized and cannot serve as an energy source for growth (*153–155*). Further, in this organism

added α-ketoglutarate remained unlabeled when isotopic acetate was oxidized while added succinate, malate, and fumarate became radioactive (*156*) The analysis of the labeling pattern of intracellular intermediates by Krampitz and his collaborators (*156–158*) has shown in several bacteria (as in the case of yeast) that these findings can be

TABLE III
BACTERIA IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE TRICARBOXYLIC ACID CYCLE HAS BEEN OBTAINED

Organism	References
Aerobacter aerogenes	Wong and Ajl (*153*), Gilvarg and Davis (*154*)
Acetobacter pasteurianum	King, *et al* (*155*)
Acetobacter peroxidans	Atkinson (*156*)
Alcaligenes faecalis	Tourtellotte *et al* (*157*)
Azotobacter vinelandii	Stone and Wilson (*158*)
Bacillus cereus	Beck and Lindstrom (*159*)
Bacillus subtilis	Wiame and Bourgeois (*160*)
Brucella abortis	Altenbern and Housewright (*161*)
Corynebacterium creatinovorans	Fukui and Vandemark (*162*)
Escherichia coli	Swim and Krampitz (*149*), Gilvarg and Davis (*154*)
Micrococcus lysodeikticus	Saz and Krampitz (*163*)
Mycobacterium phlei	Blakley (*164*)
Mycobacterium tuberculosis	Millman and Youmans (*165*)
Pasteurella pestis	Englesberg and Levy (*166*)
Propionibacterium pentosaceum	Delwiche and Carson (*167*)
Pseudomonas aeruginosa	Campbell and Stokes (*168*)
Pseudomonas fluorescens	Barrett and Kallio (*169*), Kogut and Podoski (*170*)
Rhodopseudomonas capsulatus	Stoppani *et al* (*171*)
Rhodospirillum palustris	Crook and Lindstrom (*172*)
Rhodospirillum rubrum	Crook and Lindstrom (*172*)
Staphylococcus aureus	Stedman and Kravitz (*173*)

accounted for by permeability barriers The intracellular metabolites become labeled on addition of C^{14}-acetate as expected, but some of the di- and tricarboxylic acids do not mix with the added metabolites in the medium This has been established, among others for *Micrococcus lysodeikticus* (*147*) and *Escherichia coli* (*148*).

The nature of the permeability barrier which separates internal and external metabolites, has recently been investigated by Campbell and Stokes (*168*) [illegible]

of certain metabolites into the cell has been shown to depend on the presence, within the cell, of specific proteins which act as transporting

agents, even against concentration gradients. Many observations on adaptation to the use of tricarboxylic acid cycle intermediates, can be explained by the formation of "permeases." For example *Aerobacter* can utilize added citrate provided the organism has been grown in the presence of citrate If the organism is grown on glucose or succinate it does not metabolize citrate immediately but does so after an exposure of at least 30 minutes This adaptation to citrate cannot represent a new formation of enzymes which degrade citrate, since such enzymes are known to be already present before adaptation They can be demonstrated in extracts, and they are known, from isotope studies, to take part in the synthesis of glutamate from sugar or lactate, by the route shown in Diagram B.

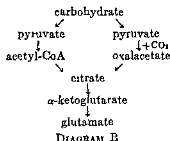


DIAGRAM B

It follows that the "adaptation" to citrate utilization means the elaboration by the cells of a mechanism which renders the external citrate accessible to the enzymes which, in the unadapted cells, act on the endogenous citrate.

D. OBSERVATIONS ON MOLDS

Within the last ten years data have been reported which indicate the occurrence of the tricarboxylic acid cycle in many species of molds A list of different types of organisms in which the occurrence of the cycle has been studied is given in Table IV.

Citric acid is a major end product of the oxidative metabolism of carbohydrate, ethanol, and acetic acid in many molds, e.g., *Aspergillus niger*, and as early as 1919 Raistrick and Clark (*178*) suggested that citric acid might be formed by a reaction involving a condensation of oxalacetate with "active" acetate. The newer work has conclusively confirmed the concept (*179–181*). Experiments with isotopic CO_2 and isotopic glucose suggest that the oxalacetate required for the synthesis of citrate can be formed by the carboxylation of pyruvate arising as an intermediate in the anaerobic fermentation of carbohydrate (*179–184*). It is obvious that there must be another mechanism of oxalacetate formation when

C_2-compounds like ethanol and acetate are the sole sources of carbon. One such other mechanism—probably the main one—is the glyoxylate cycle discussed in Section V,A This cycle may also account for the formation of fumaric acid by *Rhizopus nigricans* (*185*) and possibly of α-ketoglutarate by *Aspergillus niger* (*186*) and *Penicillium chrysogenum* (*187*).

TABLE IV
MOLDS IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE TRICARBOXYLIC ACID CYCLE HAS BEEN OBTAINED

Organism	References
Ashbya gossypii	Mickelson and Schuler (*188*)
Aspergillus niger	Ramakrishnan (*189*)
Neurospora crassa	Lewis (*190*), Strauss (*191*)
Penicillium chrysogenum	Goldschmidt *et al* (*192*)
	Hockenhull *et al* (*193*)
Streptomyces griseus	Gilmour *et al* (*194*)
Streptomyces nitrificans	Schatz *et al* (*195*)
Zygorrhynchus moelleri	Moses (*196*)
Allomyces macrogynus	Bonner and Machlis (*197*)

IV. The Cycle in Plant Material

A INTRODUCTION

The difficulties which have hampered progress in the study of the respiration of microorganisms, especially those arising from the existence of permeability barriers, were also encountered in plant material The relative slowness of metabolic processes in many plant tissues provided additional complications These difficulties have been overcome since 1951 when Millerd *et al* (*198*) succeeded in preparing mitochondria from bean seedlings and demonstrated the occurrence of oxidative reactions in the isolated mitochondria This made it possible to study respiration *in vitro* under controlled conditions without interference from major permeability barriers such as the outer cell wall.

The tricarboxylic acid cycle was first considered as the terminal pathway of oxidation in plants by Chibnall (*199*), who attempted to correlate observations on the changes of the "plant acids" and "plant amides" (asparagine and glutamine) He pointed out in 1939 that the cycle could account for many observations, but added "the scheme must be regarded at present as nothing more than a convenient, if speculative, working hypothesis" Vickery and Pucher (*200*), surveying the data on the concentration of organic acids in rhubarb leaves at different stages

of culture, came to the conclusion in 1940 that no observation was in conflict with the assumption that the tricarboxylic acid cycle is operative.

Thunberg (*201*) was the first to discover that plant materials in particular seeds and also pollen (*202*), contain a set of dehydrogenases similar to that found in animal tissue.

From 1946 onward experiments on a variety of plant materials such as cut-up spinach leaves (*203*), segments of oat coleoptiles (*204*), segments of roots of barley seedlings (*205*), and slices of potato tubers revealed that many characteristics of the oxidative processes in these materials are similar to those in animal tissues. The intermediates of the tricarboxylic acid cycle were found to be readily oxidized and malonate proved to be a specific inhibitor.

B. Observations on Mitochondrial Preparations

One of the decisive experiments of Millerd *et al.* (*198*) demonstrating the reactions of the cycle in the water-insoluble particles from etiolated

TABLE V

OXIDATIONS BY WASHED CYTOPLASMIC PARTICLES[a] OF ETIOLATED MUNG BEAN SEEDLINGS (*Phaseolus aureus*) (*198*)

Additions to medium	O_2 consumption ($\mu l\ O_2$/mg N/hr)
None	0
Citrate	208
α-Ketoglutarate	220
Succinate	202
Fumarate	23
L-Malate	54
L-Malate ($1.7 \times 10^{-3}\ M$)	7
Pyruvate	0
Pyruvate, L-malate ($1.7 \times 10^{-3}\ M$)	105

[a] Washed particles suspended in medium containing 0.3 *M* sucrose, 0.05 *M* phosphate buffer, $5 \times 10^{-4}\ M$ ATP, and $10^{-3}\ M$ $MgSO_4$, 30°, 0.02 *M* substrate unless stated otherwise, total volume 1.5 ml.

bean seedlings, is shown in Table V. Millerd *et al.* made use of the experience gained in the handling of animal tissues. This had shown that the medium used for separating the intracellular organelles must have a high osmotic pressure and that the temperature at which the mitochondria are isolated must be close to 0°. The concentration of orthophosphate was also found to be critical in this material. As seen in Table V, there was no significant blank oxidation. Citrate, α-ketoglutarate, and succinate were rapidly oxidized, whereas the oxidation of malate and fumarate

was slow when these substances are added alone Pyruvate is not attacked at all when added as the sole substrate but a mixture of pyruvate and malate causes a rapid oxygen uptake Malate was found to be replaceable by other members of the cycle Low concentrations of these substances ($1\ 7 \times 10^{-3}\ M$) were effective, indicating that their action is catalytic

TABLE VI
MITOCHONDRIAL PLANT PREPARATIONS IN WHICH EVIDENCE FOR THE OCCURRENCE OF THE TRICARBOXYLIC ACID CYCLE HAS BEEN OBTAINED

Plant material	References
Cauliflower buds	Laties (*220*)
Etiolated mung bean seedlings	Millerd (*221*), Bonner and Millerd (*222*)
Sweet potato	Akazawa and Uritani (*223*), Lieberman and Biale (*239*)
Arum spadix	Hackett and Simon (*224*), James and Elliott (*225*), Simon (*226*), Bendall (*227*)
Potatoes	Sharpendsteen and Conn (*228*), Barron *et al* (*206*)
Etiolated oat coleoptile	Tager (*229*)
Etiolated bean hypocotyls	Beaudreau and Remmert (*230*)
Lupine cotyledons	Conn and Young (*231*)
Broccoli buds	Lieberman and Biale (*232*)
Spinach leaves	Ohmura (*233*)
Lettuce seedlings	Poljakoff-Mayber (*234*)
Pea seedlings, green and etiolated (roots, stems, leaves)	Smillie (*235*); Davies (*236*)
Green pea leaves	Smillie (*237*)
Castor bean endosperm	Beevers and Walker (*238*)
Sugar pine, seed and seedlings	Stanley (*240*), Stanley and Conn (*241*)
Avocado fruit	Avron and Biale (*242*)
Unicellular algae	Calvin and Benson (*217*, *218*), Calvin and Massini (*219*)

The lower rate of oxidation of malate and fumarate is probably connected with the fact that the oxidation of malate is inhibited by oxalacetate and unless the latter is removed, e g , by the formation of citrate, the oxidation of malate is inhibited

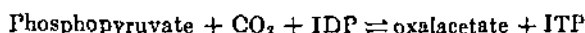
The oxygen consumption of the bean mitochondria is coupled with the phosphorylation of ADP, as is the case in the mitochondria of animal tissues

Subsequently particles prepared from other materials proved to possess similar characteristics A list of species and materials which have been studied are shown in Table VI Some differences, e g , in the phosphate requirement, have been encountered but in general the similarity

of the mitochondria of different origin is striking. For the details, the reader is referred to reviews by Goddard and Stafford (*207*), Hackett (*208*), and Laties (*209*)

C. Observations on Living Plants

Experiments on the behavior of isotopic carbon compounds in intact plant material bear out the occurrence of the tricarboxylic acid cycle Thus, leaves of Crassulaceae (*Bryophyllum calicinum*) on brief exposure to $C^{14}O_2$ (6 seconds) in the dark rapidly incorporate the isotope into malate and aspartate (*210*) The common precursor of these two substances is oxalacetate formed by the carboxylation of phosphopyruvate (*211–213*):



$$\text{Phosphopyruvate} + CO_2 + \text{IDP} \rightleftharpoons \text{oxalacetate} + \text{ITP}$$

On more prolonged incubation all the intermediates of the tricarboxylic acid cycle and the amino acids related to it become labeled (*214*). Green algae, such as *Chlorella* or *Scenedesmus*, incorporate labeled acetate and labeled CO_2 very quickly into the intermediates of the cycle and the related amino acids (*215–219*)

V. Modified Tricarboxylic Acid Cycles

A The Glyoxylate Cycle

The glyoxylate cycle (*243–247*) came to light in the search for a mechanism by which carbon chains are synthesized in those organisms which can derive all their energy and carbon requirements from acetate or ethanol Yeasts, many molds, *Escherichia coli*, and pseudomonads belong to this group Experiments in which cells of a *Pseudomonas* strain were exposed to (C^{14})-acetate and the appearance of labeled compounds was traced by paper chromatography and autoradiography showed that most of the intermediates of the tricarboxylic acid cycle became labeled within minutes (*244*). By shortening the period of incubation it was not possible to reduce the label to mainly one compound (as had been possible in Calvin's experiments on photosynthesis). The data on the time course of the isotope distribution indicated that acetate entered the tricarboxylic acid cycle simultaneously at two points to form citrate at one and a C_4-dicarboxylic acid at another· at the earliest stages after addition of (C^{14})-acetate (3 seconds) the radioactivity of malate was much higher than expected on the basis of the assumption that malate was solely formed via citrate by the reactions of the tricarboxylic acid cycle Experiments on cell extracts confirmed the conclusion that acetate can enter the cycle by two different reactions. With acetyl coenzyme A and oxal-

acetate the extracts, like those from many other tissues and cells, form citrate.



$$\text{Acetyl CoA} + \text{oxalacetate} \rightarrow \text{citrate} + \text{CoA}$$

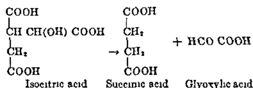
With acetyl coenzyme A and glyoxylate they form L-malate:



$$COOH\ CHO + CH_3\ CO\ S\ R \rightarrow COOH\ CH(OH)CH_2\ COOH + HS\ R$$

These two reactions are closely analogous in that in both cases the methyl group of acetyl coenzyme A condenses with the carbonyl group of an α-ketonic acid, but they are catalyzed by two different enzymes, the "condensing enzyme" and "malate synthetase" The latter enzyme was first discovered by Wong and Ajl (*248*) in extracts of *E. coli*

The glyoxylate required for the malate synthetase reaction is supplied by the "isocitritase" reaction, first observed (*249–254*) in *Pseudomonas* in 1953



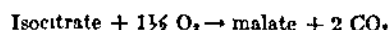
Isocitric acid Succinic acid Glyoxylic acid

When this enzyme was first encountered, it was thought (*253*) that its function was to supply the glyoxylate for the synthesis of glycine, and in reverse to provide the cyclic mechanism for the biological oxidation of compounds more highly oxidized than acetate, such as glycine or glycollate Experiments by Kornberg and Madsen (*246, 255*) demonstrate that the glyoxylate formed by the action of isocitritase readily reacts with acetyl coenzyme A to form malate When cell-free extracts of *Pseudomonas* are provided with isocitrate and acetyl coenzyme A, malate and succinate are formed, and the stoichiometry of the reaction is as expected:



$$\text{Isocitrate} + \text{acetate} \rightarrow \text{succinate} + \text{malate}$$

Thus the combined action of isocitritase and malate synthetase can replace the steps of the tricarboxylic acid cycle leading from isocitrate to malate In the tricarboxylic acid cycle these steps are oxidative:



$$\text{Isocitrate} + 1\tfrac{1}{2}\ O_2 \rightarrow \text{malate} + 2\ CO_2$$

whereas the combined action of isocitritase and malate synthetase is an anaerobic process. It follows that a variant of the tricarboxylic acid cycle can take place in the organism studied which employs most of the steps

of the original cycle but substitutes the reactions of isocitritase and malate synthetase for the degradative steps between isocitrate and malate The net effect of one turn of this "glyoxylate cycle," shown in Fig. 5, is the synthesis of one molecule of succinate from two molecules of acetate

The occurrence of a reaction leading from two molecules of acetate to succinate was postulated long ago and has often been discussed, in particular by Thunberg (*2*) and by Knoop (*4*). These authors assumed a

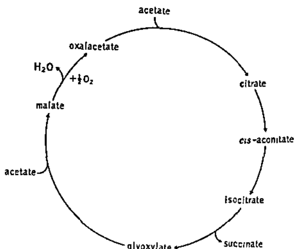


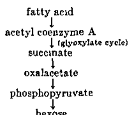
FIG 5 The main stages of the "glyoxylate cycle" Acetate reacts in the form of acetyl coenzyme A The net effect of one turn of the cycle is 2 acetate + ½ O_2 → succinate + H_2O

rather direct condensation of two molecules of acetate coupled with dehydrogenation It is now clear that this condensation can be achieved by an entirely different mechanism involving the intermediary stages of the glyoxylate cycle

This cycle accounts for the net synthesis of C_4-carboxylic acids from acetate when acetate is the sole source of carbon It can therefore supply the oxalacetate required for the continuous operation of the tricarboxylic acid cycle Moreover, since C_4-dicarboxylic acid can serve as starting material for the synthesis of many cell constituents, e g , carbohydrate, amino acids, pyrimidines, it is evident that the glyoxylate cycle can serve as a stage in the synthesis of cell constituents from C_2-compounds

The reactions of the glyoxylate cycle have been demonstrated in many types of microorganisms, e g , various pseudomonads (*247*), *E coli* (*256*), *Aerobacter* (*252*), coryneform bacteria (*247*), bakers' yeast, and other species of *Saccharomyces* (*257*), *Aspergillus* (*258*), and *Penicillium* (*259*).

The glyoxylate cycle is also relevant to the problem of the biological conversion of fat to carbohydrate. Since C_4-dicarboxylic acids formed from acetate by the glyoxylate cycle can be converted to phosphopyruvate and thence to sugar, it follows that this cycle could act as a link in the formation of carbohydrate from fatty acids, the stages being



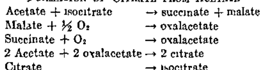
A conversion of fat to carbohydrate is known to occur in seedlings rich in oil, as are those of the castor bean (*Ricinus communis*) (*260*), pumpkin (*Cucurbita pepo*) (*261*), sunflower (*Helianthus annuus*) (*262*), and peanut (*Arachis*) (*262*) Cell-free extracts of seedlings of these plants were in fact found to contain the two characteristic enzymes of the glyoxylate cycle, viz, isocitritase and malate synthetase, and to be able to catalyze the formation of malate from acetate and isocitrate (*260–262*) The other reactions required for a conversion of fat to carbohydrate are known to occur in plant material These findings support the assumption that the glyoxylate cycle provides a link in the net conversion of fat to carbohydrate

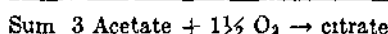
It is uncertain to what extent this occurs in higher animals The search for the specific enzymes of the glyoxylate cycle in animals has so far been negative (*263*).

There is another series of observations which can be satisfactorily accounted for by the glyoxylate cycle A number of microorganisms oxidize organic substances incompletely in such a way that intermediates of the tricarboxylic acid cycle appear as major end products of metabolism. Thus *Aspergillus* (*180*) produces citric acid from sugars or acetic acid, the mold *Rhizopus* (*185, 264*) forms fumaric acid from sugars or ethanol or acetic acid, several microorganisms, especially the genera *Pseudomonas* (*265, 170*) and *Vibrio* (*266–271*), accumulate α-ketoglutaric acid from sugars, succinate, fatty acids, or acetate It has long been appreciated that the reactions of the tricarboxylic acid cycle must play a role in the accumulation of these substances, but it was also clear that the cycle alone cannot explain the accumulation, because the continuous occurrence of the cycle depends on the oxidation of the intermediates to the stage of oxalacetate If the oxidation is interrupted, the cycle

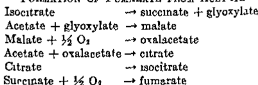
must come to a standstill unless oxalacetate is provided from another source. Evidently the glyoxylate cycle represents such another source. The presence of enzymes of this cycle in molds and bacteria, and isotope data on the distribution of radioactive carbon (*180, 185, 264*) are in accordance with the assumption that the glyoxylate cycle takes part in the reactions leading from acetate to citrate and to fumarate in the following way.

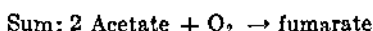
FORMATION OF CITRATE FROM ACETATE


Acetate + isocitrate → succinate + malate
Malate + ½ O_2 → oxalacetate
Succinate + O_2 → oxalacetate
2 Acetate + 2 oxalacetate → 2 citrate
Citrate → isocitrate


Sum 3 Acetate + 1½ O_2 → citrate

FORMATION OF FUMARATE FROM ACETATE


Isocitrate → succinate + glyoxylate
Acetate + glyoxylate → malate
Malate + ½ O_2 → oxalacetate
Acetate + oxalacetate → citrate
Citrate → isocitrate
Succinate + ½ O_2 → fumarate


Sum: 2 Acetate + O_2 → fumarate

In both cases, it will be noted, isocitrate acts as a "catalyst" It initiates the sequence of reactions but is later regenerated and thus becomes available for another set of reactions

There is an alternative reaction leading to oxalacetate, provided that glucose, or another sugar, or a suitable 3-carbon compound is the source of carbon In this case, oxalacetate is formed by interaction between pyruvate and CO_2 Many observations (*180*) with isotopic CO_2 have shown the occurrence of this reaction, though its mechanism is not yet fully established

While malate synthetase appears to be a constitutive enzyme, isocitritase is an adaptive enzyme which is not formed when glucose or succinate are present in the growth medium Thus the glyoxylate cycle does not operate when there is no need for the synthesis of succinate, either because it is supplied in the medium, or because it is formed by another route

B THE CYCLE OF GLYCINE OXIDATION

Since glycine is more highly oxidized than acetic acid it is not possible for a glycine derivative to enter the tricarboxylic acid cycle without a series of special preparatory steps One of these is the conversion into serine by aldol condensation with formaldehyde, the latter reacting in an

"active" form. The ready interconversion of glycine and serine has been conclusively established (see Volume II, Chapter 15). It is thus possible that a major part of glycine shares the pathway of degradation with serine and yields acetyl coenzyme A.

Another possible pathway of glycine degradation, suggested by Shemin (*272, 273*) is initiated by the condensation of glycine with succinyl

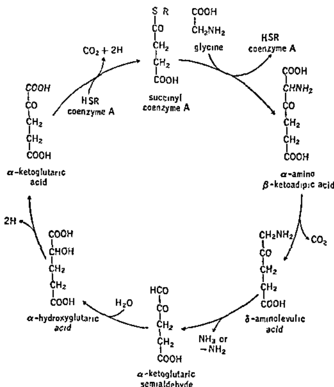


FIG 6 The cycle of glycine oxidation Shemin (*272, 273*)

coenzyme A according to the scheme shown in Fig 6 The conversion of glycine and succinyl coenzyme A to δ-aminolevulic acid has been firmly established (*274–277*), and the occurrence of two of the other steps has been demonstrated in various animal tissues These are the transamination reaction of δ-aminolevulic acid (*278*) and the oxidation of α-hydroxyglutarate to α-ketoglutarate (*106, 279*). The one remaining reaction is the dismutation of α-ketoglutaric semialdehyde The postulated reaction is analogous to the conversion of methylglyoxal to lactic acid, as catalyzed by glyoxalase (*260*)

Thus the cycle can be regarded as a feasible pathway but to what extent if any it actually serves as a route of glycine degradation is ... tain. Th... glycin... "activ... formaldehyde The sources of the latter are limited.

C. Glutamic-Aspartic and Aspartic-Glutamic Cycles

When glutamic acid is the substrate added to tissue slices, homogenates, or washed particles, a major oxidative reaction taking place

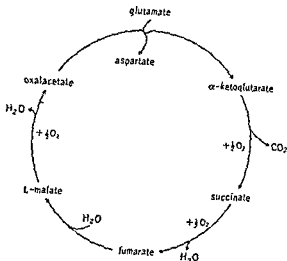
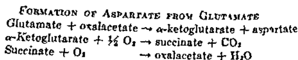
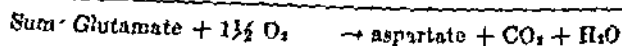


Fig 7 The glutamic-aspartic cycle (*Net effect*: Glutamate + $1\frac{1}{2}$ O_2 → aspartate + CO_2 + H_2O.)

is the conversion of glutamate to aspartate (*231*). This is due to a modified tricarboxylic acid cycle (Fig. 7). glutamate is oxidized via the stages of the tricarboxylic acid cycle to oxalacetate. Most of the oxalacetate then reacts by transamination with a second molecule of glutamate to form aspartate and α-ketoglutarate. The balance of this sequence is as follows:


Formation of Aspartate from Glutamate

Glutamate + oxalacetate → α-ketoglutarate + aspartate
α-Ketoglutarate + $\frac{1}{2}$ O_2 → succinate + CO_2
Succinate + O_2 → oxalacetate + H_2O


Sum: Glutamate + $1\frac{1}{2}$ O_2 → aspartate + CO_2 + H_2O

Some oxalacetate may undergo decarboxylation and complete oxidation via pyruvate and acetyl coenzyme A but in the presence of an excess of

glutamate the oxidation of glutamate is incomplete and "cut short" by the transamination reaction In pigeon liver, pigeon breast muscle, or rat brain, 50–100% of the amino nitrogen of glutamate can be recovered as aspartate *(282)*

Similarly the oxidation of aspartate leads to an accumulation of glutamate *(281, 283)* This is due to a somewhat different modification of the tricarboxylic acid cycle (Fig 8) The α-ketoglutarate formed as an

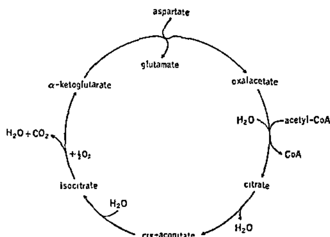
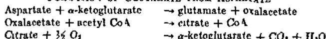


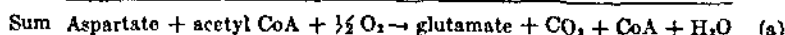
FIG 8 The aspartic-glutamic cycle (Net effect Aspartate + acetyl CoA + $\frac{1}{2}O_2 \rightarrow$ glutamate + CO_2 + CoA)

intermediate is side-tracked by the transamination reaction with aspartate and appears as glutamate The balance of this sequence is as follows·

FORMATION OF GLUTAMATE FROM ASPARTATE

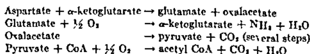


Aspartate + α-ketoglutarate	→ glutamate + oxalacetate
Oxalacetate + acetyl CoA	→ citrate + CoA
Citrate + $\frac{1}{2}O_2$	→ α-ketoglutarate + CO_2 + H_2O

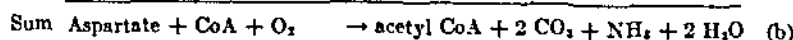


Sum Aspartate + acetyl CoA + $\frac{1}{2}O_2 \rightarrow$ glutamate + CO_2 + CoA + H_2O (a)

In addition aspartate can also give rise to acetyl coenzyme A by the following reaction sequence.

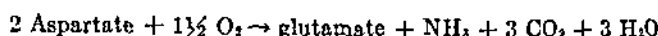


Aspartate + α-ketoglutarate	→ glutamate + oxalacetate
Glutamate + $\frac{1}{2}O_2$	→ α-ketoglutarate + NH_3 + H_2O
Oxalacetate	→ pyruvate + CO_2 (several steps)
Pyruvate + CoA + $\frac{1}{2}O_2$	→ acetyl CoA + CO_2 + H_2O



Sum Aspartate + CoA + O_2 → acetyl CoA + 2 CO_2 + NH_3 + 2 H_2O (b)

In the absence of other substrates two molecules of aspartate can therefore give rise to one molecule of glutamate as is shown by taking the sum of (a) and (b):

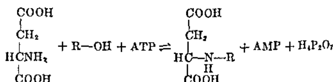


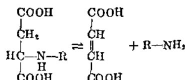
$$2 \text{ Aspartate} + 1\frac{1}{2}\, O_2 \rightarrow \text{glutamate} + NH_3 + 3\, CO_2 + 3\, H_2O$$

The aspartic-glutamic transaminase, being of exceptionally high activity, is the main transaminase which can interfere with the tricarboxylic acid cycle in a major way. Other transaminases, such as the alanine-glutamic transaminase, are too weak to cause an appreciable diversion of the cycle

D. The Aspartic Cycles

The amino groups of a number of compounds are derived from aspartate by a two-step reaction of the type





Quantitatively the most important reaction of this kind in the animal is the amination of citrulline to yield arginine, one of the key steps in the urea cycle (*284–286*). Another is the amidation of 5-amino-4-carboxy iminazole ribotide to yield 5-amino-4-carbox-amino iminazole ribotide (*287, 288*), one of the steps in the synthesis of purines. A third example is the amination of inosinic acid leading to adenylic acid (*289–291*). The aspartate used up in these reactions is regenerated by the reoxidation of the resul ... ation of the latter with glut ... skeleton

VI. The Role of the Tricarboxylic Acid Cycle in the Synthesis of Cell Constituents

The tricarboxylic acid cycle, in conjunction with ancillary reactions, also plays an important role in the synthesis of cell constituents from the simple carbon compounds contained in the food or nutrient media.

The most widespread ancillary reaction occurring in many types of living cells is the formation of C_4-dicarboxylic acids from pyruvate and carbon dioxide, catalyzed by the malic enzyme or the oxalacetate carboxylase (see page 181) In microorganisms and some plant material the glyoxylate cycle is, under certain conditions, a further essential link in the synthesis of cell constituents

There are three α-ketonic acids which are intermediates in the tricarboxylic acid and glyoxylate cycles; oxalacetate, α-ketoglutarate, and glyoxylate A fourth α-ketonic acid, pyruvate, arises by the decarboxylation of oxalacetate, or as an intermediate of glycolysis These four

TABLE VII

INTERMEDIATES OF THE TRICARBOXYLIC ACID AND GLYOXYLATE CYCLES AS PRECURSORS OF THE CARBON SKELETON OF CELL CONSTITUENTS*

(The substances marked with an asterisk are not synthesized *de novo* in higher animals, neither does the glyoxylate cycle operate in higher animals)

Starting materials	Products synthesized
α-Ketoglutarate	Glutamate, proline, hydroxyproline, arginine,* citrulline,* ornithine*
Succinyl-CoA	Porphyrins
Oxalacetate	Aspartate, pyrimidines, threonine,* isoleucine,* phosphopyruvate, hexoses, hexosephosphate, pentose phosphates, phenylalanine,* tyrosine,* tryptophan
Glyoxylate	Glycine, purine bases, porphyrins, serine

* Evidence for the occurrence of the reactions listed in this table is given in the following selected papers (*120, 293 294*) (animal tissues), (*295*) (*E coli*, *Neurospora Chlorella*, *Torulopsis*), (*296, 297, 298 299*) (yeast), (*160*) (*B subtilis*) (*177*) (*E coli*, Aerobacter), (*300, 301*) (general review)

α-ketonic acids form the corresponding α-amino acids—aspartate, glutamate, alanine, and glycine—either by transamination or by reductive amination Aspartate, glutamate, and glycine in turn are precursors of many other amino acids as well as of purines, pyrimidines, and porphyrins Other important starting materials for syntheses are oxalacetate which, via phosphopyruvate can give rise to hexoses, pentoses, and the carbon skeletons of the aromatic amino acids, and succinyl-CoA which participates in the synthesis of porphyrins Since the pathways of these syntheses are fully discussed in other sections of this book, a brief summary of the role of the tricarboxylic acid cycle and its ancillary reactions in the synthesis of cell constituents, as given in Table VII, will here suffice

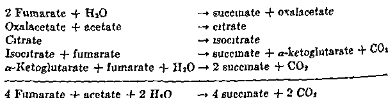
The evidence in support of the statements contained in Table VII rests on the demonstration of the enzyme systems required for the interconversions, and on isotope data obtained with many kinds of materials.

VII. The Cycle under Anaerobic Conditions

Although molecular O_2 is not *directly* concerned with the enzymic mechanisms of the tricarboxylic acid cycle the working of the cycle depends under most physiological conditions on the continuous supply of O_2. The reason is that the four dehydrogenase systems of the cycle lead to the formation of reduced cofactors, and unless these are reoxidized the cycle must come to a standstill.

There are, however, special circumstances where O_2 can be replaced as terminal hydrogen acceptor by normally occurring metabolites. An example is furnished by *Escherichia coli* in media containing an excess of fumarate. This organism readily reduces the fumarate to succinate when conditions are anaerobic (*302, 149*). Many substrates can serve as the hydrogen donors. In the case of acetate the hydrogen required for the reduction of fumarate arises in the three stages of the tricarboxylic acid cycle providing reduced pyridine nucleotides (*149*). The fourth dehydrogenation of the cycle, that of succinate, cannot take place anaerobically under normal conditions because of the position of the redox potential of the succinate-fumarate system which makes a major net hydrogen transfer from succinate to physiologically occurring substances (except to O_2 and catalysts of the respiratory chain) impossible.

Quantitative measurements of the chemical changes and isotope data agree with the view that the following anaerobic substrate changes take place in *E. coli* in the presence of fumarate and acetate (*302, 149*):



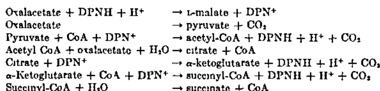
2 Fumarate + H_2O	→ succinate + oxalacetate
Oxalacetate + acetate	→ citrate
Citrate	→ isocitrate
Isocitrate + fumarate	→ succinate + α-ketoglutarate + CO_2
α-Ketoglutarate + fumarate + H_2O	→ 2 succinate + CO_2
4 Fumarate + acetate + 2 H_2O	→ 4 succinate + 2 CO_2

These reactions represent coupled oxido reductions, in which fumarate acts as the chief hydrogen acceptor, the oxidative reactions of the tricarboxylic acid cycle between malate and succinate act as hydrogen donors, and pyridine nucleotides act as the chief intermediary hydrogen carriers. Of the four succinate molecules arising from fumarate, three are formed by reduction of fumarate and one by oxidation of acetate plus fumarate. The two molecules of CO_2 formed per molecule of acetate removed are derived from the added fumarate, while the carbon of acetate appears in the succinate molecule as Swim and Krampitz (*149*) have shown with the help of C^{14}-acetate.

These anaerobic reactions in *E. coli* can be rapid and of the same order as the aerobic oxidation of acetate (*302*) This does not hold for animal tissues where the reduction of fumarate is extremely slow, if it occurs at all Differences in the rate of reduction of fumarate by succinic dehydrogenase between animal tissues and microorganisms appear to be paralleled by the behavior of the highly purified enzymes the succinic dehydrogenase of *Micrococcus lactilyticus* (*Veillonella gazogenes*) readily reduces fumarate in the presence of a suitable hydrogen donor (reduced methylviologen) while enzyme preparations from animal tissues do not (*303, 99, 101*)

Similar anaerobic reactions take place in *E coli* in the presence of oxalacetate Again part of the added substrate is reduced to succinate while another undergoes oxidation (*302*).

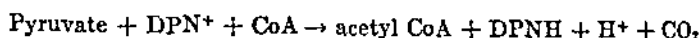
Unlike fumarate, oxalacetate is reduced also in animal tissues, the main end product of the reduction being malate and fumarate This reduction is accompanied by the formation of citrate, α-ketoglutarate, succinate, and carbon dioxide (*304, 305*) The main anaerobic reactions which take place on addition of oxalacetate to a variety of animal tissues can be formulated as follows


Oxalacetate + DPNH + H^+ → L-malate + DPN^+
Oxalacetate → pyruvate + CO_2
Pyruvate + CoA + DPN^+ → acetyl-CoA + DPNH + H^+ + CO_2
Acetyl CoA + oxalacetate + H_2O → citrate + CoA
Citrate + DPN^+ → α-ketoglutarate + DPNH + H^+ + CO_2
α-Ketoglutarate + CoA + DPN^+ → succinyl-CoA + DPNH + H^+ + CO_2
Succinyl-CoA + H_2O → succinate + CoA

These represent the stages of the tricarboxylic acid cycle between oxalacetate and succinate via the tricarboxylic acids, plus the reduction of oxalacetate to malate The rate of these reactions can be very high In pigeon breast muscle, for example, the rate of hydrogen transfer to oxalacetate, given an excess of oxalacetate, is of the same order as the rate of hydrogen transfer to O_2 in maximum respiration (*304*) No substrate other than oxalacetate can be reduced at comparable speeds on addition to animal tissues A reduction of *added* pyruvate or α-ketoglutarate, it is true, can occur but the rates of these reductions are much slower This is not due to the low activity of the dehydrogenases catalyzing the reduction, but to the lack of an oxidative reaction suitable for coupling The [illegible]

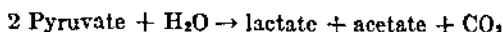
When *added* pyruvate is reduced the triose phosphate dehydrogenase

reaction must be replaced by another reaction of pyruvate, such as



$$\text{Pyruvate} + DPN^{+} + \text{CoA} \rightarrow \text{acetyl CoA} + DPNH + H^{+} + CO_2$$

This oxidative reaction requires several cofactors in catalytic amounts, among them DPN and CoA, and unless the CoA is regenerated from acetyl CoA the above reaction comes to a stop Hence the dismutation

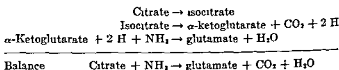


$$2\ \text{Pyruvate} + H_2O \rightarrow \text{lactate} + \text{acetate} + CO_2$$

can occur only if acetyl CoA is deacetylated. Specific deacetylating enzymes appear to be present in staphylococci, gonococci, many other microorganisms, and in a few animal tissues as indicated by the occurrence of the dimutation of pyruvate (*306*) but they are absent from others such as muscle In many animal tissues CoA can be regenerated from acetyl CoA by the action of the condensing enzyme if oxalacetate is available This is an essential link in anaerobic reactions of oxalacetate

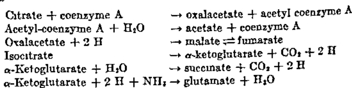
The rapid reduction of oxalacetate in animal tissue, first observed by Szent-Gyorgyi, led earlier workers (*11*, *307*) to assume that the reversible system malate ⇌ oxalacetate plays a special role of hydrogen transport in animal tissues In the light of the newer knowledge it appears that the rapid reduction may be coincidental, being the result of the availability of suitable oxidative reactions which can be coupled with reduction of oxalacetate

Several animal tissues (liver, kidney, brain, heart muscle) reduce α-ketoglutarate in the presence of NH_4^+ to glutamate This reductive amination can be coupled with various steps of the tricarboxylic acid cycle (α-ketoglutarate → succinate, isocitrate → α-ketoglutarate, malate → oxalacetate) (*308*). Hence citrate when added to tissue homogenates together with NH_4Cl can yield glutamate anaerobically (*64*, *309*):



	Citrate → isocitrate
	Isocitrate → α-ketoglutarate + CO_2 + 2 H
α-Ketoglutarate + 2 H + NH_3 → glutamate + H_2O	
Balance	Citrate + NH_3 → glutamate + CO_2 + H_2O

Washed particles from rat liver metabolize citrate anaerobically to malate, fumarate, α-ketoglutarate, glutamate, acetate, and succinate (*310*) The data suggest the main anaerobic reactions of citrate are as follows



Citrate + coenzyme A	→ oxalacetate + acetyl coenzyme A
Acetyl-coenzyme A + H_2O	→ acetate + coenzyme A
Oxalacetate + 2 H	→ malate ⇌ fumarate
Isocitrate	→ α-ketoglutarate + CO_2 + 2 H
α-Ketoglutarate + H_2O	→ succinate + CO_2 + 2 H
α-Ketoglutarate + 2 H + NH_3	→ glutamate + H_2O

Although the anaerobic reactions of the cycle are not accompanied by a major release of free energy, the anaerobic occurrence of reactions of the cycle is not merely a matter of theoretical interest. Apart from supplying energy the cycle can also serve to supply carbon skeletons required in growing organisms for the synthesis of cell material (see Chapter 4) This applies also to organisms capable of growing anaerobically, such as yeast, and certain steps of the cycle, such as those leading to α-ketoglutarate, are therefore essential to the growth of such organisms In this connection, it is noteworthy that anaerobically grown yeast usually contains substantial amounts (more than 1% of its dry weight) of succinate This is probably formed by anaerobic oxido-reductions of the type discussed in the preceding paragraphs

The formation of succinate as a by-product of many other fermentations may be accounted for in the same way The major reductive processes of fermentations, i e the reduction of pyruvate to lactate or of acetaldehyde to ethanol, are usually paired with the oxidation of triosephosphate Hence, additional reductive reactions are required by anaerobes for those oxidations which occur in the course of the synthesis of cell constituents from sugars. Quantitatively prominent among such oxidations is the formation of glutamate from glucose This interconversion requires acceptors for three pairs of hydrogen atoms

The position of the oxidation-reduction potential of the succinate-fumarate system ($E_0' = 0\ 031$ volts at pH 7 0) is more positive than the great majority of other dehydrogenase systems, and may be of special importance in the promotion of anaerobic oxidations

In some microorganisms such as the coliforms the formation of formate (or of $H_2 + CO_2$) can act as an additional hydrogen accepting reaction It serves in particular to balance the oxidation of pyruvate to acetyl CoA.

The cycle may also be expected to occur anaerobically in those microorganisms which can reduce nitrate (*311–314*), e g , *Escherichia coli, Clostridium welchii, Proteus vulgaris, Pseudomonas aeruginosa, Micrococcus denitrificans*, or sulfate (*315*) e g , *Desulphovibrio desulphuricans*

VIII. Inhibitors of the Tricarboxylic Acid Cycle

Reference has already been made to the specific inhibition of succinic dehydrogenase by malonate In low concentrations of malonate (up to 10^{-3} *M*) succinic dehydrogenase is the only enzyme which suffers a major inhibition at physiological substrate concentrations This is borne out by the accumulation of succinate in the tissues and body fluids of animals after injection of malonate (*116, 117*).

Two other inhibitors are known which interfere specifically with

dehydrogenase reactions of the cycle, viz., fluorocitrate and parapyruvate. The inhibitory effect of fluorocitrate was discovered in the course of studies on the mechanism of action of fluoroacetate This substance was introduced as a rat poison in 1954 because of its somewhat selective toxicity for wild rodents (*316*) Bartlett and Barron (*317*) found in 1947 that it inhibits the oxidation of acetate in animal tissues and causes an accumulation of acetate in the presence of pyruvate No effects on individual stages of the tricarboxylic acid cycle were found, and Bartlett and Barron therefore considered fluoroacetate as a specific, possibly competitive, inhibitor of acetate breakdown Kalnitsky and Barron (*318*, *319*) found an accumulation of citrate in the presence of fluoroacetate in kidney homogenates and Buffa and Peters (*118*, *320*) discovered citrate *in vivo* in many tissues after the injection of fluoroacetate into rats Increases, in some cases seventyfold, occurred in kidney, heart, spleen, intestines, lung, brain, liver, and striated muscle α-Ketoglutarate did not accumulate and Buffa and Peters concluded that fluoroacetate blocks the conversion of citrate into α-ketoglutarate Liébecq and Peters (*321*), Martius (*322*), and Elliot and Kalnitsky (*323*) have suggested that fluoroacetate as such is not responsible for the accumulation of citrate, but another inhibitor arising from fluoroacetate and oxalacetate, perhaps fluorocitrate or a similar fluorotricarboxylic acid Buffa *et al.* (*324*), Lotspeich *et al* (*325*), and Peters and Wilson (*326*) in fact succeeded in separating a tricarboxylic acid fraction from tissue homogenates incubated with oxalacetate or fumarate and fluoroacetate The fraction contains small amounts of an organic fluorine compound, probably a fluorotricarboxylic acid, which inhibits the disappearance of citrate from respiring tissues homogenates The comparison of this compound with an authentic synthetic material indicated that it was fluorocitric acid (*327*) According to Morrison and Peters (*328*) fluorocitrate specifically inhibits aconitase which accounts for the accumulation of citrate There are quantitative differences between the inhibitory power of the synthetic fluorocitrate and the fluorotricarboxylic acid isolated from tissue preparations after incubation, but these differences can be explained by the assumption that the synthetic fluorocitrate may consist of two or four stereoisomers while the enzymically formed substance probably contains only one isomer

Parapyruvate (COOH.C(OH)(CH_3) CH_2.CO COOH) which is readily prepared by treating freshly distilled pyruvic acid with dry HCl (*329*) may be looked upon as a structural analog of α-ketoglutarate It inhibits specifically the oxidation of α-ketoglutarate in various types of tissue preparations (*330–332*) When pyruvate is the only oxidizable substrate added to muscle homogenates the respiration is greatly reduced by para-

pyruvate, but it is restored by the addition of succinate and fumarate Considerable quantities of α-ketoglutarate accumulate under these conditions (*332*).

There are numerous inhibitors which interfere with the cycle because they prevent the reoxidation of reduced coenzymes by molecular O_2 HCN and H_2S are prototypes of such inhibitors They act at the iron porphyrin level and stop the tricarboxylic acid cycle under normal conditions, but this inhibition may be regarded as a secondary effect. At the concentrations usually employed these substances do not interfere directly with the primary reactions of the cycle, e g , those where the substrate changes occur

Other inhibitors of hydrogen and electron transport are 2,3-mercaptopropanol (BAL) (*333–335*), antimycin A (*336–338*), and Amytal (*339*). The precise points of attack of these agents is still under discussion.

IX. Concentration of Intermediates in Respiring Cells

The intermediates of the tricarboxylic acid cycle do not normally accumulate in cells to appreciable levels Modern analytical methods

TABLE VIII
AVERAGE CONCENTRATION OF INTERMEDIARY METABOLITES IN TISSUES OF NORMAL RATS[a]

Metabolite	Concentration of metabolites ($10^4 \times M$)		
	Liver	Kidney	Muscle
Fumarate	7 3	6 1	0 5
α-Ketoglutarate	1 9	1 7	0 1
Succinate	1 5	1 5	0 08
Malate	1 1	0 8	0 07
Citrate[b]	1 2	1 3	0 005

[a] Animals fasted for 24 hours, anesthetized with Nembutal, metabolic activity of tissues stopped by cooling with dry ice

[b] See also B Buffa and R. A Peters (*118*)

nevertheless have made it possible to measure the concentrations of most of the intermediates in respiring cells Data for rat tissues given by Frohman *et al* (*340*) are shown in Table VIII. The concentrations of most of the intermediates are of the order of 10^{-4} *M* Neish (*341*) using a highly specific separation procedure found only 0 1 – 0 3 × 10^{-4} *M* α-ketoglutarate in rat tissues. The average oxalacetate level was found to be 1 × 10^{-5} *M* in rat liver (*105*) and of the same order in rat brain and kidney (*342*).

Swim and Krampitz (*148*) found the following levels in *E. coli* oxidizing acetate at 30°.

Citrate	5.2×10^{-5} *M*
α-Ketoglutarate	4.2×10^{-5} *M*
Succinate	18.4×10^{-5} *M*
Fumarate	1.8×10^{-5} *M*
L-Malate	1.5×10^{-5} *M*

Considerably higher levels have been found by DeMoss and Swim (*150*) in baker's yeast oxidizing acetate.

Citrate	0.6×10^{-3} *M*
Succinate	3.5×10^{-3} *M*
Fumarate	0.9×10^{-3} *M*
Malate	3.6×10^{-3} *M*

Still very much higher concentrations (up to 0.1 *M*) can occur in plant tissues.

It is of interest to compare the quantities present with those turned over. In rat liver ($Q_{O_2} = -12$) about 10^{-4} mole of each intermediate is formed per kilogram tissue about once in every 10 seconds, and in kidney ($Q_{O_2} = -24$) in half this time. On the assumption that there is complete mixing between the newly formed intermediate and its tissues store, it can be calculated that the average lifetime of most of the intermediates in liver and in kidney is of the order of a few seconds and in the case of oxalacetate only a fraction of a second. In *Escherichia coli* it is still less than in animal tissues.

X. Reactions Leading from Foodstuffs to the Tricarboxylic Acid Cycle

A. General Survey

The degradation of the basic constituents of the foodstuffs, as already stated in the introductory paragraph to this chapter, may be said to proceed in two major stages. In the first stage, the starting materials—the small molecules produced by the processes of digestion and consisting of hexoses, about 20 amino acids, a number of fatty acids, glycerol—are incompletely burned. The products formed, apart from carbon dioxide, and water, are in the great majority of cases substances which occur as intermediates in the tricarboxylic acid cycle. Acetic acid, in the form of acetyl coenzyme A, constitutes the main product: two-thirds of the carbon of carbohydrate and glycerol, all the carbon of the common fatty acids, and about half the carbon skeleton of amino acids yield acetyl coenzyme A. α-Ketoglutarate arises from glutamic acid, histidine,

arginine, citrulline, ornithine, proline, hydroxyproline, and probably lysine; oxalacetate from aspartate; fumarate from part of the benzene rings of tyrosine and phenylalanine; succinate from isoleucine, valine, methionine, α-amino butyric acid, propionic acid, and the three terminal carbon atoms of fatty acids with an uneven number of carbon atoms.

TABLE IX
SURVEY OF THE PRODUCTS FORMED BY THE INITIAL DEGRADATION REACTIONS[a] OF THE BASIC CONSTITUENTS OF FOODSTUFFS

Starting material	Products of initial reactions (CO_2 omitted)
Glucose, other hexoses	2 Acetyl CoA
Fatty acids (even-numbered chains of n C-atoms)	½ n Acetyl CoA
Fatty acids (uneven-numbered chains of n C-atoms)	½ $(n - 3)$ Acetyl CoA 1 Succinate (via propionyl CoA)
Glycerol, alanine, cysteine, cystine, serine	1 Acetyl CoA
Glutamic acid, histidine, arginine, ornithine, citrulline, proline, hydroxyproline	1 α-Ketoglutarate
Aspartic acid	1 Oxalacetate
Leucine	3 Acetyl CoA
Isoleucine	1 Acetyl CoA 1 Succinate (via propionyl CoA)
Valine	1 Succinate (via methylmalonyl CoA)
α-Aminobutyric acid, homoserine, homocysteine, methionine	1 Succinate (via propionyl CoA)
Glycine[b]	Acetyl CoA (via serine)
Threonine	2 Acetyl CoA (one via glycine-serine)
Lysine	α-Ketoglutarate, Acetyl CoA
Phenylalanine, tyrosine	1 Fumarate 2 Acetyl CoA
Tryptophan	1 Acetyl CoA (via alanine)[c]

[a] These reactions all lead to acetyl coenzyme A and/or the intermediates of the tricarboxylic acid cycle

[b] Glycine may also be oxidized by a special cycle (Fig 6)

[c] Other products are formed which are not oxidizable

These results are more fully discussed in other sections of this book. A survey summarizing the available information is given in Table IX.

Table IX refers in the first instance to animal tissues, although most of it also applies to microorganisms. The latter possess additional enzyme systems capable of converting to intermediates of the cycle nutrients which cannot be metabolized by higher organisms. Thus soil organisms, which can derive all their carbon and energy requirements from 2,3-butanediol or acetyl-methyl-carbinol have evolved a special cyclic

mechanism which converts these substrates quantitatively to acetate (*343, 344*).

The carbon requirements of certain bacteria, especially those belonging to the family of the Pseudomonadaceae (*345*) can be met by numerous organic substances. The pathways of oxidation are still largely unknown. Several organisms can oxidize a variety of aromatic compounds such as mandelic acid, benzoic acid, *p*-cresol, catechol, as well as polycyclic hydrocarbons such as naphthalene, anthracene, and phenanthrene. The benzene ring of the former group yields in many cases one molecule of succinate and one molecule of acetate; catechol, *cis-cis* muconic acid, and α-ketoadipic acid are intermediates (*346, 347*). The polycyclic hydrocarbons are metabolized through end-ring attack and this also leads to catechol as an intermediate (*348–351*) and thence to succinate and acetate [for reviews see refs. (*351–353*)].

B. Formation of Acetyl Coenzyme A

1. *Formation of Acetyl Coenzyme A from Pyruvate*

The reactions leading from pyruvate to acetyl coenzyme A are strictly analogous to those already described for the conversion of α-ketoglutarate to succinyl coenzyme A (page 146). This also holds true for the degradation of most other α-ketonic acids, but not for oxalacetate. Much of the pioneer work which led to the elucidation of the breakdown of α-ketonic acids was in fact carried out on pyruvate. In 1936 Peters (*354*) showed that thiamine plays a part in pyruvate oxidation in animal tissues and shortly afterward Lohmann and Schuster (*355*) identified as thiamine pyrophosphate the thermostable cofactor of yeast carboxylase, which Auhagen (*356*) had discovered in 1932. This form of thiamine was later found to be a cofactor of many other keto acid decarboxylases (*357–359*). The first to demonstrate the participation of pantothenate in the oxidation of pyruvate were Dorfman *et al.* (*360*), and Hills (*361*), working on microorganisms. After the discovery of coenzyme A it became clear that coenzyme A represented the active form of pantothenate in all types of cells. Yet another cofactor of pyruvate oxidation was discovered in 1947 by O'Kane and Gunsalus (*362*) in *Streptococcus faecalis*. This was also found to be of widespread occurrence. Its structure was established by Macchi *et al.* (*363*) as 1,2-dithiolane-3-valeric acid, and the official trivial name "lipoic acid" was adopted in 1955 [see Reed (*364*)].

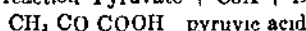
The detailed formulation of the formation of acetyl coenzyme A from pyruvate as shown in Scheme 6, is now generally accepted as the best interpretation of the experimental findings. It evolved from the work of Korkes *et al.* (*365, 366*), Korkes (*367*), Schweet *et al.* (*368*),

Littlefield and Sanadi (*369*), and Gunsalus (*75*) For a fuller discussion of the reaction types the reader is referred to the section on the conversion of α-ketoglutarate to succinyl-coenzyme A (page 146)

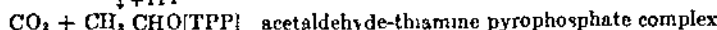
SCHEME 6

FORMATION OF ACETYL COENZYME A FROM PYRUVATE

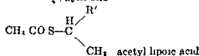
(Over-all reaction Pyruvate + CoA + $DPN^+ \rightarrow$ acetyl CoA + DPNH + H^+ + CO_2)



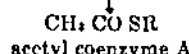
↓ +TPP



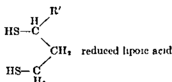
↓ + lipoic acid



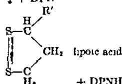




+



↓ + DPN^+



+ DPNH + H^+

It should be mentioned that there is evidence of other reactions of pyruvate by which this is converted to acetate in some microorganisms Experiments by Moyed and O'Kane (*370*) suggest that *Proteus vulgaris* forms acetate from pyruvate without requiring DPN, coenzyme A, and lipoic acid

2 *Conversion of Acetate to Acetyl Coenzyme A*

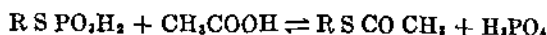
Many animal tissues, plants and microorganisms contain enzyme systems which convert acetate to acetyl coenzyme A provided that ATP, Mg^{2+} ions, and coenzyme A are present. One of the main problems concerning the mechanism of action of this "acetate-activating enzyme" is that of the role of ATP in the synthesis of acetyl coenzyme A Lynen and Reichert (*371*) pointed out that it is unlikely that ATP, acetate, and coenzyme A react together simultaneously and they suggested that two

stages may be involved, the first being a phosphorylation of coenzyme A (R SH) by ATP:



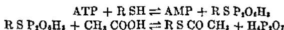
$$ATP + R\,SH \rightleftharpoons ADP + R\,S\,PO_3H_2$$

followed by the substitution of phosphate by acetate:



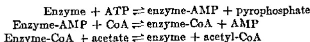
$$R\,S\,PO_3H_2 + CH_3COOH \rightleftharpoons R\,S\,CO\,CH_3 + H_3PO_4$$

However the search by Lipmann *et al.* (*372*) for the orthophosphate expected according to this scheme was unsuccessful; instead pyrophosphate was found Moreover, no ADP, but AMP was formed These observations led to the hypothesis that a pyrophosphorylated coenzyme A may be an intermediate

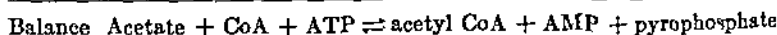


$$ATP + R\,SH \rightleftharpoons AMP + R\,S\,P_2O_6H_3$$
$$R\,S\,P_2O_6H_3 + CH_3\,COOH \rightleftharpoons R\,S\,CO\,CH_3 + H_4P_2O_7$$

But this scheme had to be abandoned also, when further joint studies by Jones *et al.* (*373*) on purified yeast enzymes showed that P^{32}-pyrophosphate readily exchanged with ATP in the absence of coenzyme A This excluded the latter as an obligatory participant in the primary reaction of ATP *These authors also found that isotopic acetate exchanges* readily with acetyl coenzyme A in the presence of the yeast enzyme To explain the observed facts they suggested the formation of enzyme-bound adenosine phosphate and coenzyme A:



Enzyme + ATP ⇌ enzyme-AMP + pyrophosphate
Enzyme-AMP + CoA ⇌ enzyme-CoA + AMP
Enzyme-CoA + acetate ⇌ enzyme + acetyl-CoA



Balance Acetate + CoA + ATP ⇌ acetyl CoA + AMP + pyrophosphate

This scheme became untenable when Berg (*374*) demonstrated that the enzyme preparations used by Jones *et al* contained traces of acetate, and that this was essential for the exchange reaction between pyrophosphate and ATP He further found that acetylhydroxamic acid is formed from ATP, acetate, and hydroxylamine by the highly purified yeast enzyme in the absence of coenzyme A; that the exchange of C^{14} AMP with ATP is dependent on the presence of acetate and coenzyme A; and that the exchange reaction between ac[illegible] ...tyl coenzyme A requires AMP and [illegible] te These ob[illegible] led to the conclusion that the prim[illegible] formation [illegible] A from acetate is the formation of [illegible] (acetyl ade[illegible] in a second step interacts with co[illegible]

This mechanism was finally clinched by the demonstration that synthetic acetyl AMP is converted to ATP in the presence of pyrophosphate, and to acetyl CoA in the presence of CoA

The mechanism of carboxylic acid "activation" discovered by Berg has since been shown to apply also to the activation of fatty acids (*375–377*), the α-carboxylic group of amino acids (*374, 378*) and certain aromatic acids (*379, 380*). It is not fully understood why the "activation" of carboxylic acids requires a fission of the β rather than the terminal (γ) group of ATP A possible reason is that more energy is required for the activation of acetate than is liberated by the fission of the terminal pyrophosphate bond of ATP While the *standard* free energies of hydrolysis of the two pyrophosphate bonds of ATP are almost equal (as shown by the equilibrium position of the adenylate kinase reaction) the actual free energy change may be considerably greater for the β pyrophosphate bond This is because the concentrations of AMP and pyrophosphate in the tissues are usually much lower than those of ADP and orthophosphate Any pyrophosphate formed is quickly hydrolyzed by the powerful pyrophosphatases of the tissues

3 *Other Reactions Leading to Acetyl Coenzyme A*

The main other sources of acetyl coenzyme A are fatty acids and ketone bodies The formation of acetyl coenzyme A from these precursors is dealt with in other sections of this treatise

C CO_2-Fixing Reactions Leading to the Synthesis of C_4-Dicarboxylic Acids

1. *Introduction*

CO_2 fixation by heterotrophic organisms was discovered in 1936 by Wood and Werkmann (*381*), in the course of a study of the fermentation of glycerol by propionic acid bacteria The end products of this fermentation—mainly propionic and succinic acids—were found to contain more carbon than had been added in the form of glycerol. This extra carbon proved to come from the calcium carbonate which had been added in order to neutralize the acids formed during the fermentation. The amounts of carbon dioxide used and succinate formed were approximately equimolar (*382*), and Wood and Werkmann put forward the hypothesis that the synthesis of succinate involves a formation of oxalacetate from pyruvate and CO_2 and the reduction of oxalacetate to succinate (*383*) In 1940 evidence was obtained that a somewhat similar process took place in pigeon liver (*15, 384*) and the use of isotopic substrates subsequently established the capacity of many animal tissues, microorganisms and

plant materials to incorporate CO_2 into the carboxyl group of C_4-dicarboxylic acids. The CO_2-fixation reaction leading from pyruvate to C_4-dicarboxylic acids was referred to as the "Wood-Werkmann reaction." Earlier work in this field has been fully reviewed by Werkmann and Wood (*385*) and Utter and Wood (*386*).

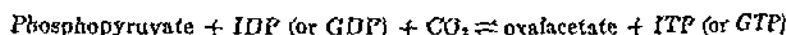
Within the last ten years the "Wood-Werkmann reaction" has been found to be due to at least three separate enzymic mechanisms. These are:

(a) the reversible reaction catalyzed by the "malic enzyme":



$$\text{Pyruvate} + CO_2 + \text{TPNH} + H^+ \rightleftharpoons \text{malate} + \text{TPN}$$

(b) the reversible reaction catalyzed by the "oxalacetate synthesizing enzyme":



$$\textit{Phosphopyruvate} + \textit{IDP} \text{ (or } \textit{GDP}\text{)} + CO_2 \rightleftharpoons \text{oxalacetate} + \textit{ITP} \text{ (or } \textit{GTP}\text{)}$$

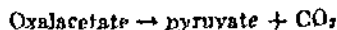
(c) the enzyme system bringing about the carboxylation of propionate



$$\text{Propionate} + CO_2 \rightarrow \text{succinate}$$

That more than one CO_2 fixation mechanism might exist was first suggested by Vennesland *et al.* (*387*) in 1947 and finally clinched in 1951 by Utter (*388*) who demonstrated two different mechanisms for CO_2 fixation by pyruvate in pigeon liver. One involves the malic enzyme and requires TPN but no nucleotide triphosphate (ATP, GTP, or ITP). The other involves what is now called the "oxalacetate synthesizing enzyme" and requires nucleotide triphosphate but no TPN. When purified extracts are incubated with pyruvate and $C^{14}O_2$ the isotope appears in malate before it appears in oxalacetate provided that TPN is present. But when TPN is replaced by ATP or ITP the isotope appears first in oxalacetate. Utter and Kurahashi later completely separated the two enzyme systems.

A further enzyme related to this group is the "oxalacetic carboxylase" which catalyzes the reaction



$$\text{Oxalacetate} \rightarrow \textit{pyruvate} + CO_2$$

It is however doubtful whether oxalacetic carboxylase is a separate enzymic entity. It cannot be separated from the "malic enzyme" and may be identical with it, or an essential component of it, except in a few microorganisms such as *Micrococcus lysodeikticus* where Herbert (*389*) found a specific oxalacetic carboxylase free from the malic enzyme. It is unlikely that oxalacetic carboxylase plays a role in the net fixation of CO_2, except as a component of the "malic enzyme," but it may be of

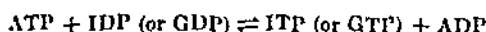
importance in the breakdown of oxalacetate when this arises as an intermediate in the combustion of aspartate and other substrates.

2. *The Malic Enzyme*

The malic enzyme was first described in pigeon liver by Ochoa *et al.* (*390*), and has since been found in many other animal tissues (*391*), [brain, heart muscle, striated muscle, retina, lens (*392*)], in the roots and leaves of plants (*391, 393, 394*) (parsley, beet, carrot), in wheat germ (*391*), and in microorganisms (*189, 246, 395–397*) The reaction catalyzed by the enzyme is reversible, but the high value of the TPNH.TPN ratio in the cytoplasm favors the synthesis of malate, rather than its breakdown The TPNH used in the reaction is regenerated by other dehydrogenase reactions Liver homogenates thus readily transform pyruvate to malate The enzyme is inhibited by a number of dicarboxylic acids (*395–398*), e.g., oxalacetate, malonate, oxalate, tartronate, mesoxalate, mesotartrate, and fluoromalate (*399*)

3 *The Oxalacetate Synthesizing Enzyme*

A formation of phosphopyruvate from C_4-dicarboxylic acids was first observed by Kalckar (*400*) in kidney preparations and later studied by Leloir and Munoz (*401*) and by Bartley (*402*) and Bartley and Avi-Dor (*403*) in liver and kidney The intermediary stages of the formation of phosphopyruvate were established by Utter and Kurahashi (*404*), who discovered the reversible interconversion of phosphopyruvate and oxalacetate in the livers of pigeons and chickens Bandurski and Lipmann (*405*) and Kurahashi *et al* (*406*) established the reaction to be specific for inosine and guanosine phosphates Preparations of ATP and ADP are usually also active because of the contamination of adenosine phosphates with inosine phosphates, and the action of nucleoside diphosphokinase (*407, 408*), which catalyzes transphosphorylations between nucleotides:



$$\text{ATP} + \text{IDP (or GDP)} \rightleftharpoons \text{ITP (or GTP)} + \text{ADP}$$

An enzyme similar to that in animal tissues has been found in wheat germ (*409*) This material also contains, as do spinach leaves, a different type of oxalacetate synthesizing enzyme It utilizes phosphopyruvate and CO_2, but does not require a nucleoside diphosphate as phosphate acceptor Orthophosphate is formed and the reaction is practically irreversible (*409, 410*)

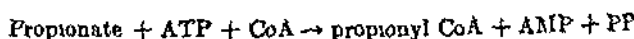
In the direction from right to left the reaction of Utter and Kurahashi is an important link in the synthesis of carbohydrate from C_4-dicarboxylic acids, from C_3-compounds, and from acetate (*411, 412*) In the direction

from left to right the reaction converts phosphopyruvate formed by glycolysis to C_4-dicarboxylic acids. It can therefore provide the oxalacetate required for the operation of the tricarboxylic acid cycle, and act as a link in the synthesis of the carbon skeleton of certain amino acids from carbohydrates.

4 *Propionyl Coenzyme A Carboxylase*

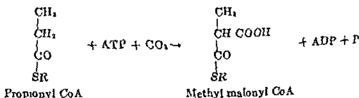
Propionate has long been known (*413, 414*) to be glycogenic in animals but the pathway of the conversion of propionate to carbohydrate has only recently become clear when Lardy (*413, 414*) and Friedberg *et al.* (*415*), showed that liver mitochondria contained enzymes which synthesize succinate from propionate and bicarbonate. An important further step in the elucidation of the mechanism of action of these enzymes was the discovery in 1955 by Flavin *et al.* (*416*) and independently by Katz and Chaikoff (*417*), that methyl malonate (isosuccinate) is an intermediate in the formation of succinate from propionate.

The details of this metabolic process have been clarified by Ochoa and his collaborators (*418–421*) The first step is the formation of propionyl coenzyme A·

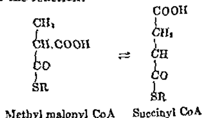


Propionate + ATP + CoA → propionyl CoA + AMP + PP

This is followed by a CO_2 fixation reaction requiring ATP:

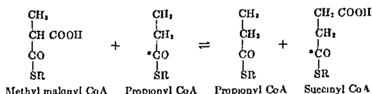


The next step, catalyzed by "methyl malonyl coenzyme A iso (*420*), brings about the reaction:



This is probably not a rearrangement within one molecule but a

carboxylation" involving the transfer of the carboxyl group from methyl malonyl CoA or succinyl CoA to propionyl CoA (*421*):



Methyl malonyl CoA Propionyl CoA Propionyl CoA Succinyl CoA

This is supported by the fact that incubation of the purified isomerases with propionyl CoA labeled with C^{14} in the propionyl molecule and unlabeled methyl malonyl CoA (or succinyl CoA) yields labeled succinyl CoA (or methyl malonyl CoA). No labeled products are formed when unlabeled methyl malonyl CoA or succinyl CoA are omitted, or when unlabeled propionyl CoA is incubated in the presence of C^{14}-labeled bicarbonate (*421*).

The last step in the formation of succinate is the deacylation of succinyl CoA.

Succinyl CoA and propionyl CoA may also be concerned with the interconversion of succinate and propionate in propionic acid bacteria (*422*, *423*) and in *Micrococcus lactilyticus* (*Veillonella gazogenes*) (*423*) but there is as yet no evidence of the intermediate formation of methyl malonyl CoA in these microorganisms.

XI. Control of the Rate of the Tricarboxylic Acid Cycle

As cell respiration is a complex process its rate can be affected by numerous different factors. These include the concentrations of enzymes, of cofactors, and of substrates. At several levels specific control mechanisms operate which adjust the rate to changing needs. One of the control mechanisms is concerned with the over-all rate of oxygen consumption. This rate depends on the speed at which electrons are transferred from the reduced catalysts of the respiratory chain to molecular oxygen. In many respiring cells electron transport is obligatorily coupled with the phosphorylation of ADP, hence the reduced electron carriers cannot be reoxidized—at least not at the full rate—unless ADP and phosphate are available. This is a feedback control system: the expenditure of energy by the fission of ATP exerts a direct control over the rate of oxygen consumption. [For a fuller discussion of this subject see (*349*, *422–427*).]

The rate of electron transport controls the over-all rate of substrate

oxidation and indirectly also the rate of the tricarboxylic acid cycle Of more direct concern to the control of the cycle is another aspect of the regulation of respiration, viz, the choice of substrate when a variety of substances are available. The oxidized carriers can accept electrons from many of the starting materials of respiration (e g., lactate, glutamate, or fatty acids) as well as from intermediate metabolites, (e.g, pyruvate and the acids of the tricarboxylic acid cycle). Intermediates do not normally accumulate This indicates that the electron carriers react with the intermediates in preference to reacting with the starting materials In other words, once the oxidation of a substrate molecule has been initiated it burns to completion before a new molecule is attacked *Thus the rate-limiting step of the tricarboxylic acid cycle must be that which* initiates the cycle, i.e, the reaction between oxalacetate and acetyl coenzyme A. This conclusion is confirmed by the experience that the addition of the cycle intermediates to respiring cells often increases the rate of oxygen consumption, as well as the rates of the individual reactions of the cycle This is taken to mean that the enzymes responsible for the steps of the cycle are not used to full capacity when the normal endogenous stores serve as substrates for respiration Citrate therefore reacts as rapidly through the cycle as it arises from oxalacetate and acetyl coenzyme A

These considerations lead to the question of what controls the rate of formation of citrate A priori, either the amounts of enzyme, or of oxalacetate, or of acetyl coenzyme A may be expected to be rate-limiting It is not possible yet to state to which extent these three factors take part in the rate control It appears that the amounts of condensing enzyme are not a controlling factor in many situations This may be inferred from the fact that the rates may increase when an organ changes from the resting state to activity, or when the two substrates of the condensing enzyme are added

An important point bearing on this problem is the accumulation of acetate, in the form of ketone bodies, in the ketotic liver and the nonaccumulation of acetate or ketone bodies in normal tissues As already stated nonaccumulation indicates that the removal of intermediates is as rapid as their formation, and it follows that the rate of formation of citrate, and hence of the tricarboxylic acid cycle, is controlled by the rate at which acetyl coenzyme A becomes available from its precursors

This cannot apply to the exceptional case of liver cells in the ketotic state when more acetyl coenzyme A is produced than can be metabolized through the tricarboxylic acid cycle (or through the various anabolic processes such as the synthesis of fatty acids or steroids). The excess of acetyl coenzyme A accumulates in the form of the ketone bodies. This is one of the few exceptional situations where intermediates accumulate in

relatively large amounts[2] What limits the rate of the tricarboxylic acid cycle in the ketotic liver is not yet understood Determinations of oxalacetate suggest that its level is not the limiting factor (*105*) Nor can it be explained why there is an overproduction of acetyl coenzyme A in the ketotic organism, i e, why the formation of acetyl coenzyme A is not controlled by the rate of its disappearance through the tricarboxylic acid cycle as is normally the case The control mechanisms probably involve hormones since the formation of ketone bodies can be influenced by the hormones of the pituitary and adrenal cortex (*428*) The mechanism of action of these hormones is obscure, their point of attack is very probably the rate of acetyl coenzyme A formation, rather than of acetyl coenzyme A utilization

XII. The Free Energy Changes Associated with the Individual Steps of the Cycle[3]

As the liberation of energy is the major physiological function of the tricarboxylic acid cycle the quantitative aspects of the free energy changes associated with the individual steps of the cycle are of wide interest The collection of data in this field began some twenty-five years ago Progress was slow at first but has been more rapid in recent years (*429–431*) Reasonably accurate free energy data are now available for many of the steps of the cycle and related reactions (*432, 433*) They are summarized in Table X

The reader is referred to Chapter 1 "Free Energy and Metabolism" for a general treatment of the thermodynamic principles involved, knowledge of which is presumed in the following discussion Attention is also drawn to articles by Gillespie *et al* (*434*), Kalckar (*435*), and Burton (*436*), for the clarification of various special aspects

Free energy changes depend on the concentrations, or more precisely the thermodynamic activities, of the reactants According to the accepted convention ΔF^0 refers to idealized standard activities, $\Delta F'$ to specially specified standard activities and ΔF to the actually prevailing activities[4] The relations between ΔF^0 and ΔF are expressed by the equation

$$\Delta F = \Delta F^0 + RT \ln \frac{\text{product of activities of reactants on right hand side}}{\text{product of activities of reactants on left hand side}}$$

[2] "Intermediates" here refers to the metabolism of the body as a whole

[3] This section is by K Burton and H A Krebs

[4] Free energy at constant temperature and pressure (Gibbs free energy) has usually been denoted by the symbol *F* in the American literature (see G N Lewis and M Randall, "Thermodynamics," McGraw-Hill, New York, 1923), whereas the symbol "*G*" has been recommended by the Royal Society Committee on Symbols (1951) and has been much used in Great Britain.

TABLE X

FREE ENERGY CHANGES OF THE INDIVIDUAL STEPS OF THE TRICARBOXYLIC ACID CYCLE AND ASSOCIATED REACTIONS[a]

Reactions	K^b	$\Delta F'$	ΔF
Tricarboxylic acid cycle			
Oxalacetate^{2-} + acetyl CoA + H_2O → citrate^{3-} + CoA + H^+	5×10^5	−7.8	−7.8
Oxalacetate^{2-} + acetate → citrate^{3-}	0.5	+0.4	+3.1
Citrate^{3-} → *cis*-aconitate^{3-} + H_2O	0.07	+2.0	+2.0
cis-Aconitate^{3-} + H_2O → isocitrate^{3-}	2	−0.4	−0.4
Isocitrate^{3-} + ½ O_2 + H^+ → α-ketoglutarate^{2-} + CO_2 + H_2O		−54.9	−56.9
α-Ketoglutarate^{2-} + ½ O_2 + CoA + H^+ → succinyl CoA$^-$ + CO_2 + H_2O		−60.7	−60.0
Succinyl CoA$^-$ + MgADP$^-$ + HPO_4^{2-} → succinate^{2-} + MgATP^{2-} + CoA	4	−0.8	−0.8
Succinate^{2-} + ½ O_2 → fumarate^{2-} + H_2O		−36.2	−35.7
Fumarate^{2-} + H_2O → malate^{2-}	4.5	−0.9	−0.9
Malate^{2-} + ½ O_2 → oxalacetate^{2-} + H_2O		−45.2	−44.8
Glyoxylate cycle			
Isocitrate^{3-} → glyoxylate$^-$ + succinate^{2-}	0.07	+2.1	−0.62
Acetyl CoA + glyoxylate$^-$ + H_2O → malate^{2-} + CoA + H^+	10^8	−11.0	−11.0
Associated reactions			
Lactate$^-$ + 3 O_2 + H^+ → 3 CO_2 + 3 H_2O		−319.8	−321.7
Lactate$^-$ + ½ O_2 → pyruvate$^-$ + H_2O		−46.6	−46.1
Pyruvate$^-$ + 2½ O_2 + H^+ → 3 CO_2 + 2 H_2O		−273.2	−275.6
Pyruvate$^-$ + ½ O_2 + CoA + H^+ → acetyl CoA + CO_2 + H_2O		−61.6	−62.3
Acetaldehyde + ½ O_2 + CoA → acetyl CoA + H_2O		−56.6	−53.4
Alanine + ½ O_2 → pyruvate$^-$ + NH_4^+		−43.1	−45.3
Aspartate$^-$ + ½ O_2 → oxalacetate^{2-} + NH_4^+		−42.1	−44.3
Glutamate$^-$ + ½ O_2 → α-ketoglutarate^{2-} + NH_4^+		−43.2	−45.4
Acetoacetate$^-$ + succinyl CoA$^-$ → acetoacetyl CoA + succinate^{2-}	260	−3.3	−3.3
Succinyl CoA$^-$ + H_2O → succinate^{2-} + CoA + H^+	5×10^6	−7.8	−10.5
Acetyl CoA + H_2O → acetate$^-$ + CoA + H^+	10^6	−8.2	−10.9
ATP^{4-} + H_2O → ADP^{3-} + HPO_4^{2-} + H^+	2×10^6	−8.6	−11.3
$MgATP^{2-}$ + H_2O → $MgADP^-$ + HPO_4^{2-} + H^+	2×10^5	−7.0	−9.7
ADP^{3-} + H_2O → AMP^{2-} + HPO_4^{2-} + H^+	5×10^5	−7.8	−10.5
Acetyl phosphate^{2-} + H_2O → acetate$^-$ + HPO_4^{2-}	5×10^7	−10.5	−13.5
DPNH + H^+ + ½ O_2 → DPN^+ + H_2O		−52.4	−51.9
TPNH + H^+ + ½ O_2 → TPN^+ + H_2O		−52.6	−52.1
DPNH + cytochrome *c* (2 Fe^{3+}) → DPN^+ + reduced cytochrome *c* (2 Fe^{2+}) + H^+	2×10^{19}	−26.4	−26.4
TPNH + cytochrome *c* (2 Fe^{3+}) → TPN^+ + reduced cytochrome *c* (2 Fe^{2+}) + H^+	3×10^{19}	−26.6	−26.6

TABLE X (*Continued*)

Reactions	K^b	$\Delta F'$	ΔF
Succinate^{2-} + cytochrome *c* (2 Fe^{3+}) → fumarate^{2-} + reduced cytochrome *c* (2 Fe^{2+}) + $2H^+$	3×10^7	−10.2	−10.2
Reduced cytochrome *c* (2 Fe^{2+}) + $2H^+$ + ½ O_2 → cytochrome *c* (2 Fe^{3+}) + H_2O	10^{19}	−26.0	−25.5

[a] The standard conditions for $\Delta F'$ are 1 atm for O_2 and CO_2, the pure liquid for H_2O, 10^{-7} for the H^+ ion activity, and unit activity in aqueous solution (i.e., ideal *M*) for the other reactants. The conditions for ΔF are 0.2 atm for O_2, 0.05 atm for CO_2, the pure liquid for H_2O, 10^{-7} H^+ ion activity and 0.01 *M* for the other reactants. For ionized compounds, the standard concentration is unit activity of the ion specified irrespective of the amounts of other ions which may be present. The superscript zero as in ΔF^0 is not used because it strictly applies to unit H^+ ion activity, however ΔF^0 is equal to $\Delta F'$ if the H^+ ion does not enter into the reaction as formulated.

[b] *K* is the equilibrium constant of the reaction at pH 7. For references to sources see (*432, 435*).

Further, there is a simple relation between the standard free energy changes and the equilibrium constant *K* of a reaction

$$\Delta F^0 = -RT \ln K^{[5]}$$

It follows that ΔF is a way of indicating how far a set of concentrations is removed from the equilibrium position. ΔF is negative if the equilibrium is in the direction of the reaction proceeding from left to right and posi*tive if the equilibrium is in the opposite direction.* It is zero if the system is already at equilibrium. ΔF also supplies information on the maximum amount of useful work which can be derived by the operation of the reaction at specified concentrations, the maximum amount being equal to $-\Delta F$. This information is of importance in connection with the performance of mechanical or electrical work by a chemical system.

ΔF^0 and ΔF of a reaction can be evaluated in two main ways, either by measuring the equilibrium of the reaction, or by determining the free energies of formation of the individual reactants from heats of combustion and heat-capacity measurements of the pure compounds. For most enzyme-catalyzed reactions, ΔF can be obtained more easily from the equilibrium constants. As it has become general practice to measure equilibria as part of the characterization of the individual enzyme systems, data are now available for the equilibrium constants of all but one of the steps of the cycle (the exception being the step α-ketoglutarate → succinyl-CoA) and for many associated reactions. Furthermore, the free energies of formation of several metabolites, including acetic, fumaric, succinic, aspartic, and glutamic acids are available from heats of com-

[5] When ΔF^0 is expressed in kcal and $\log_{10}$ is used, this equation becomes, at 25°C $\Delta F^0 = -1.36 \log_{10} K$.

bustion and heat-capacity measurements. By combining the various data, ΔF can be calculated for several reactions which are not readily reversible and for which equilibrium measurements are therefore lacking. These reactions include the conversion of α-ketoglutarate to succinyl CoA, of pyruvate to acetyl CoA, the decarboxylation of oxalacetate to pyruvate, and the formation of malate from acetyl CoA and glyoxylate.

From the same thermochemical and equilibrium data it is also possible to calculate the free energies of formation for many substances for which suitable direct thermochemical measurements are not available. The standard free energies of formation ($\Delta F^0{}_f$) of some substances of biological interest are listed in Table XI. These provide a convenient basis for calculating ΔF^0 values for many conceivable reactions since ΔF^0 is equal to the sum of the $\Delta F^0{}_f$ values for the products minus the sum of the $\Delta F^0{}_f$ values for the initial reactants.

The ΔF^0 values that are in common use in chemical thermodynamics refer to standard conditions which are far removed from those usually prevailing in biological systems (e.g., the thermodynamic activities for all dissolved reactants including the H^+ ion are assumed to be unity, i.e. ideal M concentrations). The free energy changes given in Table X refer to different standard conditions which are more useful in relation to biological material. $\Delta F'$ applies to an H^+ ion activity of 10^{-7} while other reactants are taken to be in the conventional thermodynamic states. ΔF applies to conditions which are closer to the physiological state with reference to the concentrations of all reactants (0.2 atm O_2; 0.05 atm CO_2; pH 7, and 0.01 M of other reactants except water where the pure liquid is taken to be the standard state). In calculating the values attention has been paid to the binding of reactants by divalent cations (Mg^{++} and Mn^{++}) present during the measurement of the equilibrium. Corrections have therefore been applied to the data for the dehydrogenation and decarboxylation of isocitrate and for the reactions involving ATP.

The accuracy of the data can often be judged by comparing values obtained from independent sets of measurements. The differences found are usually less (in many cases considerably less) than 0.5 kcal. This is the order of error to be anticipated on account of differences of ionic strength and temperature in the various equilibrium measurements or of uncertainties in the heats of combustion and heat capacities. A somewhat greater error may be attached to the value for the malate synthetase reaction.

It should be stressed that even the modified standard conditions used for the calculation of ΔF in Table X may still be far removed from those occurring under physiological conditions. Nevertheless the differ-

TABLE XI
STANDARD FREE ENERGIES OF FORMATION
(ΔF^0_f) FROM THE ELEMENTS
(25°, Aqueous solution unless otherwise stated)

Substance	ΔF^0_f (kcal)	Substance	ΔF^0_f (kcal)
Acetaldehyde	−33.4	Glycollate⁻	−125
Acetic acid	−91.7	Glyoxylate⁻	−110
Acetate⁻	−88.2	H_2 (gas)	0.0
Acetoacetate⁻	−118	H^+	0.0
Acetone	−38.5	OH^-	−37.6
cis-Aconitate³⁻	−220.1	H_2O_2	−32.7
L-Alanine	−88.7	Lactate⁻	−123.5
NH_4^+	−19.0	L-Leucine	−85.2
L-Asparagine	−125.9	Mannitol	−225.3
L-Aspartic acid	−172.8	Malate²⁻	−202.0
L-Aspartate⁻	−167.5	Methanol	−41.9
n-Butyrate⁻	−84.3	Oxalate²⁻	−161.3
CO_2 (gas)	−94.3	Oxalacetate²⁻	−190.5
HCO_3^-	−140.3	α-Ketoglutarate²⁻	−190.7
Citrate³⁻	−278.8	O_2 (gas)	0.0
Isocitrate³⁻	−277.2	N_2 (gas)	0.0
Ethanol	−43.4	Pyruvate	−113.4
Formaldehyde	−31.2	Sorbitol	−225.3
Fructose	−218.8	Succinate²⁻	−165.0
Fumarate²⁻	−144.4	Sucrose	−370.9
Galactose	−220.7	L-Tyrosine	−92.5
Glucose	−219.2	Urea	−48.7
L-Glutamic acid	−172.4	Uric acid	−85.3
L-Glutamate⁻	−166.5	Urate⁻	−77.9
L-Glutamine	−125.4	L-Valine	−86.0
Glycerol	−116.8	Water (liquid)	−56.7
Glycine	−89.3	Xanthine	−33.3
Glycogen ($C_6H_{10}O_5$)	−159		

ence between standardized and actual ΔF values are relatively small if the ΔF values are *numerically large* as in the reactions which involve molecular oxygen For example, a thousand-fold change in the pyruvate concentration would cause a change of only 2% in ΔF for the complete oxidation of pyruvate A ten-fold change of either oxygen or carbon dioxide tens[illegible]
to fumarate [illegible]
the three re[illegible]
the thermo[illegible]
important The position is however quite different in the case of reactions with numerically smaller ΔF values, e g, those in which DPN, TPN, or

ATP are reactants. These compounds may be extensively bound to other components of the cell, e g , ATP to bivalent cations and pyridine nucleotides to enzyme proteins. There may also be appreciable concentration differences within the different cell compartments (e g mitochondria and cytoplasm) which would mean different ΔF values at different sites

Inspection of Table X shows that the greatest decreases of free energy are associated with those reactions in which oxidation by molecular oxygen occurs These are the stages which are coupled, by unknown mechanisms, to reactions by which pyrophosphate bonds of ATP are synthesized from ADP and orthophosphate (see Chapter 2). The standardized ΔF values in Table X of some of the nonoxidative stages—e g, those catalyzed by fumarase and aconitase—are low, but under actual conditions they are likely to be lower still and to be near zero If the activities of fumarase and aconitase are very high in relation to the amounts of substrates arising, the concentrations of the reactants will always remain near the equilibrium position and hence no appreciable amounts of energy will be lost at these stages.

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References to work published after March 1, 1959 may be found in the *Annual Review of Biochemistry, Annual Review of Microbiology*, and *Advances in Enzymology*

Other Pathways of Carbohydrate Metabolism

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I. Introduction

For many years, the major, if not the sole, pathway for the conversio of glucose to lactic acid was considered to be that elaborated as a resul of the classic work of Embden, Meyerhof, Parnas, and Warburg The details of this scheme have been presented in the chapter on Glycolysis (Chapter 3) The success in analyzing the steps of this complex series of reactions stems in no small measure from the choice of yeast and muscle as the biological systems investigated Indeed the very propertie of the anaerobic production of ethanol by yeast and the accumulation of lactic acid in fatigued muscle under certain conditions ensured the presence in these biological materials of precisely those vigorous enzymic systems capable of anaerobic glycolysis As has been described, the major chemical activities of yeast during ethanol production and of muscle during contraction were found to be consistent with the expected functional behavior of this anaerobic pathway. With the discovery of the enzymes of this pathway in a wide variety of organisms, the extrapo lation was made that, as in muscle and yeast, the Embden-Meyerho scheme of glycolysis was the major pathway of glucose degradation Nevertheless, a number of facts were accumulated which suggested that there was far more to carbohydrate degradation and origin than the classic form of the reversible Embden-Meyerhof scheme. Among these were (a) the discovery of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (*1–3a*), which led the glucose molecule into unfamiliar paths, (b) the existence of pentoses, hexoses, and heptose and their derivatives such as ribose, deoxyribose, galactose, glucosamine, uronic acids, sedoheptulose, neuraminic acids, carbohydrate polymers, and numerous other compounds whose origin from any point on the classic pathway is not immediately evident; and (c) the metabolism of these substances themselves, since each can be used by one type of organism or another as a source of energy and carbon for growth. Finally, as work on the individual steps of the classic scheme proceeded in one particular tissue or organism, some significant variations on the main metabolic theme have appeared The pursuit of new sequences has been richly rewarded and has led to the discovery of "alternate" pathways

of glucose metabolism The concept of pathway is both meaningful and convenient However, it should be appreciated that "other pathways" are not metabolic aberrations They are "unconventional" only as accidents of history and of choice of experimental material. Dische, as early as 1938, showed that ribose-5-phosphate was converted to hexose phosphate by erythrocytes (3b)

II. The Pentose Phosphate Pathway

A The Over-all Oxidative Cycle

The fate which may befall a hexose molecule which is metabolized by the pentose phosphate cycle is traced out in Fig 1 All of the individual enzymic steps are thoroughly characterized and all of the intermediates are known

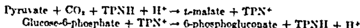
The flow diagram showing the passage of 6 molecules of hexose phosphate through the oxidative pentose phosphate cycle to give a net conversion of one hexose phosphate to CO_2 indicates the fate of the individual carbon atoms of the original hexose (Fig 2) (4).

B Enzymes of the Pentose Phosphate Pathway

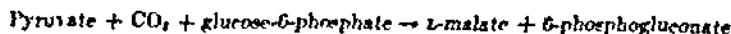
1 *Glucose-6-phosphate Dehydrogenase*

The discovery of this enzyme is of historic significance in the development of our modern knowledge of enzymology, nutrition, and intermediary metabolism. The study of the dehydrogenase from yeast led to the discovery of the coenzyme, triphosphopyridine nucleotide and subsequently to the recognition of the presence of the vitamin, nicotinamide, in TPN From this discovery has been developed the now well known and probable, but by no means completely proved, generalization which states that the activity of a vitamin is determined by its role in a coenzyme

Although the enzyme, familiarly called Zwischenferment, was stated to be widely distributed, only the material from yeast was investigated for many years. It has been used widely for the dismutation of TPN-generating systems, as in the reaction sequence:


Pyruvate + CO_2 + TPNH + H^+ → L-malate + TPN^+
Glucose-6-phosphate + TPN^+ → 6-phosphogluconate + TPNH + H^+

Over-all reaction.


Pyruvate + CO_2 + glucose-6-phosphate → L-malate + 6-phosphogluconate

The enzyme has also been useful in the estimation of the metabolite, glucose-6-phosphate (5, 6).

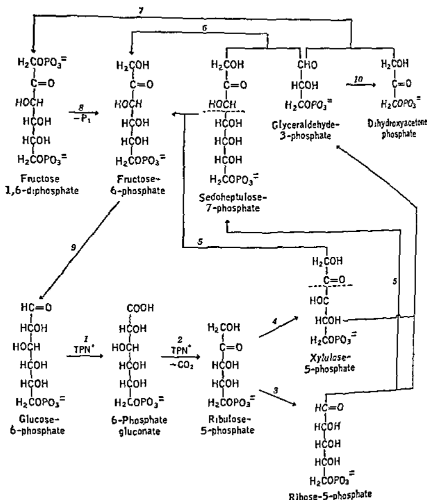


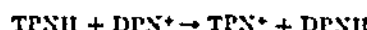
FIG 1. Reactions of the pentose phosphate cycle The individual reactions are catalyzed by the following enzymes *1* Glucose-6-phosphate dehydrogenase, *2* 6-Phosphogluconate dehydrogenase, *3* Phosphoribosisomerase, *4* D-Xylulose-5-phosphate epimerase, *5*. Transketolase, *6* Transaldolase; *7* Aldolase, *8* Phosphatase, *9* Phosphoglucoisomerase, *10* Triosephosphate isomerase.

Early work on the pathway started by this enzyme demonstrated that it led through phosphogluconate to pentose phosphate and typical products of triose phosphate degradation The presence of the enzyme has been established in yeast, *E coli* (*7–9*), *Pseudomonas* strains (*10, 11*), *B. subtilis* (*12*), and *Leuconostoc mesenteroides*, red, blue-green, and green algae (*13*), higher plants (*14–16*), the gametes of coelenterates (*17*) and

FIG 2 Schematic representation of the passage of 6 molecules of hexose phosphate through the pentose phosphate cycle The numbers refer to the positions of the carbons in the original hexose.

annelids (*13*, *18*), amphibian tissues (*13*), a wide variety of mammalian tissues (*19*), and insect tissues (*20*) and the list is constantly growing (*21a*, *21b*, *22*)

As has been stated, the enzyme of yeast is TPN-specific, as is that of *E coli* and mammalian tissues. Although the enzymes of the organisms listed above are active with TPN, it has been reported that in extracts of *Pseudomonas lindneri*, DPN is also active (*10*). It is not clear whether the coenzyme requirement in this organism is really nonspecific or that the activity of DPN may be ascribed to the reaction:



$$TPNH + DPN^+ \rightarrow TPN^+ + DPNH$$

which was first observed in *Pseudomonas fluorescens* (23). However in the case of *Leuconostoc mesenteroides* this possibility has been denied (24). In *Aspergillus flavus-oryzae* there is evidence that both TPN and DPN enzymes are present (25).

The combination of TPN with the enzyme is competitively inhibited by the sulfanilamides (7) (although this is not the basis for the antimicrobial action of this substance) and perhaps by inorganic phosphat (26), whereas the combination of the substrate with the enzyme is r ported to be competitively inhibited by atabrine (27) It has been foun that, as in many other systems, the presence of substrate stabilizes the enzyme (9)

The dehydrogenase of *E coli* has been shown to be inactive in the absence of divalent cations (9), and indeed the maximal activity of the enzyme from annelid eggs (18) or *E coli* (9) requires a relatively la amount of Mg^{++} or Ca^{++} (0 02 *M*) Assays of the enzyme have frequent been unsatisfactory because optimal ionic conditions were not provide The enzyme is inhibited by heavy metals, such as Cu^{++}. Thus the acti ity of this critical enzyme in glucose utilization is dependent on the interactions of protein, coenzyme, substrate, and divalent cations, and is sensitive to numerous inhibitory substances, some of which have been demonstrated to be active as chemotherapeutic agents

2 *6-Phosphogluconolactonase*

Evidence has been presented that the primary oxidation produc glucose-6-phosphate is the δ-lactone of 6-phosphogluconic acid (

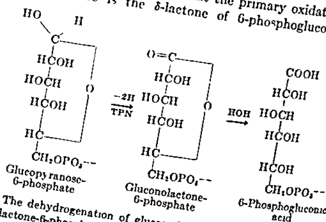


FIG 3 The dehydrogenation of glucose-6-phosphate to 6-phosphogluconic acid via gluconolactone-6-phosphate

(Fig 3) This product was observed to react rapidly with hydroxylamine to form a hydroxamic acid showing a behavior analogous to that of

δ-gluconolactone Thus glucose-6-phosphate dehydrogenase effects a dehydrogenation comparable to that produced by glucose dehydrogenase

Although the hydrolysis of the lactone to the acid can occur spontaneously at the pH optimum of the dehydrogenation, pH 7 8 to 8 7, a lactonase which catalyzes this reaction has been described. Because at times the spontaneous rate of delactonization may be inadequate, the enzyme facilitates the rapid dehydrogenation of glucose-6-phosphate

The enzymic reduction of the lactone by TPNH has been observed (*29*) However the possibility that the phosphogluconate can ultimately be converted to glucose-6-phosphate at physiological pH is limited by the fact that the lactone must first be formed

3 *6-Phosphogluconate Dehydrogenase*

a EARLY EXPERIMENTS It was shown by Warburg and his collaborators (*1, 2, 30, 31*) and Lipmann (*3a*) that enzymes of yeast using TPN as coenzyme could oxidatively decarboxylate 6-phosphogluconate The immediate reaction product was presumably pentose-5-phosphate, although it was unsatisfactorily identified by those workers It was shown, however, that the reaction need not stop at this point, involving only the consumption of 0 5 mole of O_2 and the production of 1.0 mole of CO_2, or an RQ of 2 0 Warburg and Christian obtained enzyme preparations which also went further, consuming 2 5 moles of O_2 per mole of phosphogluconate, presumably forming 3-carbon compounds In the absence of these further steps, however, reaction products were isolated, one of which analyzed as a 5-carbon compound.

This work was continued by Dickens (*32, 33*), who also used a fraction from autolyzed yeast, TPN, and as the autoxidizable hydrogen carrier, phenazine or a flavoprotein system Dickens isolated various products, one of which appeared to be a mixture of a phosphopentonic acid and a phosphoketohexonic acid, and others analyzed as 5- and 4-carbon phosphate esters The 5-carbon compound gave a reaction in the Bial $FeCl_3$-orcinol test which was characteristic of pentose. In general, the enzyme preparations of this period did not yield simple reaction products, nor were the methods available for the separation and characterization of the small amounts of products which were isolated For instance, although suggestions were obtained of the presence of pentose in the products, this was not unequivocally demonstrated

Dickens also observed the oxidation (*33*) and fermentation (*34*) by yeast systems of D-ribose-5-phosphate, but not of the expected degradation product of 6-phosphogluconate, D-arabinose-5-phosphate. He proposed that ribose arose in some manner from phosphogluconate degradation and formulated the reaction scheme of the oxidative pathway of

glucose-6-phosphate shown in Fig. 4. Despite the now obvious importance of the system, work on it virtually ceased for a decade, owing in part to the war and also in part to the difficulties involved in handling the system The early work has been reviewed by Dickens (35).

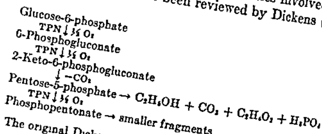


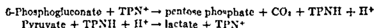
FIG 4 The original Dickens scheme of degradation of glucose-6-phosphate

b DISTRIBUTION, COENZYME SPECIFICITY, AND SOME OTHER PROPERTIES. The distribution of the dehydrogenase is essentially similar to that of the glucose-6-phosphate dehydrogenase, which is to say, that it is widely distributed. The enzyme is usually TPN specific although that obtained from *Leuconostoc mesenteroides* reduces DPN at twenty-five times the rate of TPN (*36*) In *Aspergillus flavus-oryzae* apparently t dehydrogenases are elaborated, one utilizing DPN and one TPN (*2* In general, the study of the TPN- and DPN-requiring dehydrogenase has been facilitated by following reduction of the coenzyme at 340 m in a spectrophotometer, although dye reduction methods have also proved useful (*19*) Purifications have been reported for the dehydrogenase from yeast (*37*), *E coli* (*9*), and animal tissues (*19, 38*) In *E coli*, these dehydrogenases seem to be quite soluble (*9*); in *Pseudomonas fluorescens*, the systems for glucose-6-phosphate and 6-phosphogluconate oxidation are associated with a particulate fraction (*11*). Higher plan insofar as they have been examined, contain a TPN specific enzyme (*21a*

The 6-phosphogluconate dehydrogenase also requires relatively hig concentrations of divalent cations, such as Mg^{++}, Ca^{++}, or Mn^{++} (*10, 18, 37*) Cu^{++} is strongly inhibitory, and the apparent activating effect of cyanide, glycylglycine, or protein is possibly due to the removal of heavy metal inhibitors by these agents The glucose-6-phosphate and 6-phosphogluconate dehydrogenases are not inhibited by azide, fluoride, iodoacetate, or malonate (*19*)—a matter of considerable interest with regard to reports of the lack of inhibition of respiration of some organisms in the presence of these substances.

c THE PRODUCTION AND IDENTIFICATION OF PENTOSE PHOSPHATE. A mixture of pentose phosphates has been found among the reaction products of phosphogluconate degradation The first analysis of these

products by Scott and Cohen (*39*) following the use of the enzyme preparation of Dickens revealed a small proportion of ribose-5-phosphate, the pentose of which was identified by paper chromatography and adaptive enzymic analysis (*40*). However, most of the pentose phosphate which was isolated on paper and subsequently as the free sugar was a previously uncharacterized substance Horecker and Smyrniotis, using a considerably purified enzyme preparation (*41*), found pentose phosphate was produced under conditions in which the following stoichiometry prevailed·

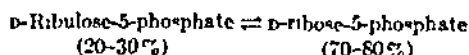


6-Phosphogluconate + $TPN^+ \rightarrow$ pentose phosphate + CO_2 + TPNH + H^+
Pyruvate + TPNH + $H^+ \rightarrow$ lactate + TPN^+

The isolated pentose phosphate proved to be predominantly D-ribose-5-phosphate in long-term experiments, and the new compound, D-ribulose-5-phosphate, in short-term experiments (*37, 42*) thus establishing that the primary product of the oxidative decarboxylation is D-ribulose-5-phosphate.

4 *Phosphoriboisomerase*

From the above results it was clear that even purified 6-phosphogluconate dehydrogenase contained a pentose phosphate isomerase which established the equilibrium:



D-Ribulose-5-phosphate $\rightleftharpoons$ D-ribose-5-phosphate
(20–30%) (70–80%)

Studies with a purified isomerase prepared from alfalfa showed essentially the same equilibria values (*43*). The equilibrium is markedly temperature-dependent owing to a relatively high entropy value ascribed to the stabilizing effect of the furanose form of the ribose phosphate

It should be noted that D-ribulose-5-phosphate is quite labile at a pH of 8, presumably as a function of its existence as a straight-chain compound (*44*) Lability of the compound does not appear to involve, in the main, an isomerization to the aldopentose phosphates, but rather an oxidative cleavage at the carbonyl group and perhaps the formation of compounds which react as enediols. It is possible that the presence of small amounts of D-arabinose isolated from reaction mixtures (*39, 45*) was due to some slight degree of isomerization. Therefore, it is evident that the characterization of D-ribulose-5-phosphate or its estimation among phosphate esters requires unusual care in minimizing its lability and the use of conditions such as a low pH or the use of acid phosphatase in subsequent dephosphorylation A derivative of ribose has not always

been used in earlier identifications of this pentose Since both pentoses contain *cis*-OH groups, they form complexes with borate and increase the acidity of borate solutions Further it has been observed that ribulose gives a distinctive color in the Bial orcinol reaction. A unique peak at 450 mμ given by the free ketopentose is almost eliminated when the compound exists as the phosphate. In this reaction ribulose gives about one-third the color at 670 mμ as the aldopentoses, all of which are approximately equally reactive A very sensitive reaction has been found for free ketopentose which is practically unaffected by the presence of a considerable excess of the aldopentoses (*44*). The ribulose isomerase to be discussed below may also be used in the specific characterization of free ribulose (*44*) Finally, ribulose is more stable than ribulose-5-phosphate to slightly alkaline conditions, although the types of changes are similar to those described above for the phosphate (*44*).

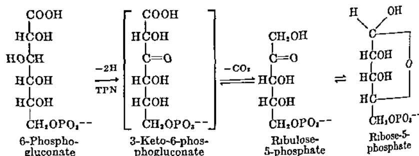


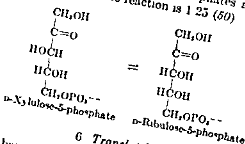
FIG 5 The Horecker scheme of conversion of 6-phosphogluconate to ribulose-5-phosphate and ribose-5-phosphate

Horecker has proposed the following reaction mechanism (Fig 5) by analogy to the oxidative decarboxylation of isocitrate via oxalosuccinate to α-keto-glutarate (*46*).

The postulated dehydrogenation at C-3 giving rise to the ketose resolves the difficulty of effecting an epimerization at C-2 of the expected precursor, D-arabinose-5-phosphate, to form D-ribose-5-phosphate However, the postulated 3-keto acid has not been isolated nor has any trace of it or its isomers been seen 3-Ketoaldonic acids have not been isolated as such; when synthesized, they are found in equilibrium mixtures with their enolic lactones Of course, it may be postulated that the intermediate 3-keto derivative is transient and perhaps stabilized at the enzyme surface Dickens and Glock have postulated the 2,3-enediol as the intermediate rather than the 3-keto acid (*19*). Neither one of these hypotheses has been proved as yet.

5. D-Xylulose-5-phosphate Epimerase

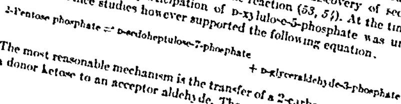
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6 Transketolase

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These workers were unable to find a 2-carbon fragment liberated i the reactions This difficulty was resolved by the discovery of sedo- eptulose-7-phosphate as a product of the reaction (*53, 54*). At the time f the discovery, the participation of D-xylulose-5-phosphate was un- nown Balance studies however supported the following equation.



2-Pentose phosphate ⇌ D-sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate

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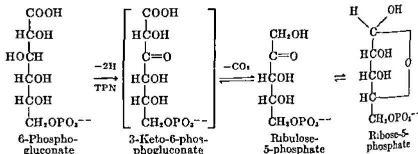


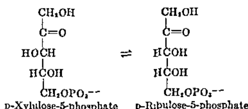
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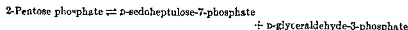
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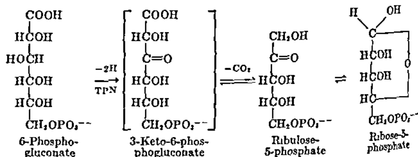


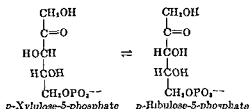
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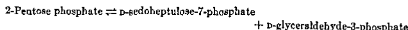


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2-Pentose phosphate ⇌ D-sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate

The most reasonable mechanism is the transfer of a 2-carbon moiety from a donor ketose to an acceptor aldehyde The moiety is apparently never

free, but must exist as enzyme-bound "active glycolaldehyde" An example of such a condensation is shown in Fig. 6. Among the active glycolaldehyde acceptors are numbered: D- and L-glyceraldehyde, glycolaldehyde, L-glyceraldehyde-3-phosphate as well as the naturally occurring metabolites, D-glyceraldehyde-3-phosphate, D-ribose-5-phosphate and D-erythrose-4-phosphate (*53–56*) In the studies made originally with crude preparations, D-ribose-5-phosphate was apparently active as a donor and acceptor, but with the availability of phosphoribоisomerase-free transketolase preparations it was established that the aldose was

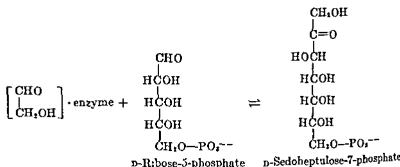
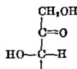


FIG 6 Postulated mechanism for the formation of sedoheptulose phosphate

not the donor It was therefore assumed that D-ribulose-5-phosphate was the true donor Other donors included D-sedoheptulose-7-phosphate, hydroxypyruvic acid, erythrulose and fructose-6-phosphate However, with the discovery of the epimerase enzyme (see above) and with the use of purer preparations of isomerase it became clear that D-ribulose-5-phosphate was not a donor. This finding cleared up the apparent anomaly of the ability of the transketolase to form condensation products with both D and L configurations at the C-3 position The specificity of the enzyme is now without exception limited to forming condensation products with the following configuration:



Conversely, only such compounds may act as donors.

Transketolase is widely dispersed throughout the biological kingdoms and has been purified from a number of sources including liver and

spinach (*54*) and has been crystallized from yeast (*55, 56*). Like the α-keto carboxylases which also catalyze active carbonyl group transfer, transketolase requires thiamine pyrophosphate and Mg^{++} (*55, 57*).

7. *Transaldolase*

Transaldolase, first found in yeast (*58*) but certainly widespread in nature, transfers the upper half of the fructose-6-phosphate molecule to a suitable aldose acceptor (Fig 7).

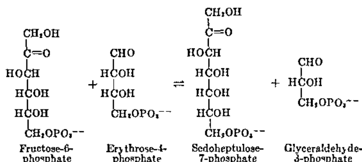
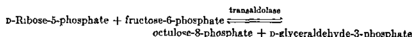


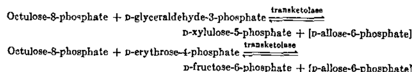
FIG 7 Action of transaldolase

Transaldolase, like transketolase, functions as a transferring enzyme; the dihydroxyacetone moiety is never free This enzyme, in concert with transketolase, is essential in the long recognized formation of hexose phosphate from pentose-5-phosphate.

A curious, if not necessarily a physiologically important result of transaldolase activity is the formation of an 8-carbon ketose as shown (*59*):



The identity of the structure of the product can be established by its behavior with transketolase and 3- or 4-carbon acceptors



In these reactions the allose phosphate remains to be identified

Muscle aldolase, exhibiting its well known tolerance for various aldehydes will also produce an octulose derivative from dihydroxyacetone phosphate and D-ribose-5-phosphate (*60*).

C The Pentose Phosphate Pathway *in Vivo*

The recognition of the pentose phosphate pathway has been followed by an avalanche of data on a myriad of organisms and tissues demonstrating both pentose phosphate enzymes and intermediates With the wide availability of tracers it has been possible to demonstrate the actual *in vivo* participation of this pathway in many tissues and studies have been undertaken with the view of assessing the relative contribution of this pathway and the Embden-Meyerhof-Parnas pathway to the metabolism of glucose

For this purpose, specifically labeled glucose, and in some cases, metabolites derived from glucose have been used. Thus in one of the earliest studies made, glucose-1-C^{14} was fed to growing *E coli* and the recovery of radioactivity as CO_2 was determined The oxidative pathway, it could be anticipated, would preferentially release C-1 while oxidation via pyruvate would favor the evolution of unlabeled CO_2 Such measurements clearly indicated that the oxidative pathway did indeed contribute to the degeneration of glucose *in vivo* (*61a*) An oft-used refinement of this procedure, that of Bloom and Stetten (*61b*), involves parallel determinations with glucose-1-C^{14} and glucose-6-C^{14} The ratio of the fractional recoveries of radioactivity in CO_2 from the two substrates provides an index of the respective roles of the two pathways, provided certain assumptions are made. Of the numerous applications of the Bloom-Stetten method only a few are mentioned as illustrations In many cases considerable uncertainty attaches to the quantitative interpretations For example, a C-6 C-1 ratio of 1.0 would result from the exclusive operation of the Embden-Meyerhof-Parnas pathway but it is conceivable that a similar value would result from the exclusive formation of CO_2 by the phosphogluconate oxidation following interchange of the upper and lower halves of the hexose by the action of aldolase. Despite the uncertainty of some results, provocative differences in the so-called C-6:C-1 ratios are observed in tissues as a function of origin, age, and various physiological conditions. Concurrent studies with labeled glycolytic intermediates, used either as starting materials or isolated after starting with specifically labeled glucose, are sometimes carried out to facilitate the interpretation. In mammalian tissues there is a suggestion that the oxidative pathway is relatively more important in mature as opposed to rapidly proliferating tissue (*61c*). In rat mammary tissue, development of the lactating state is accompanied by increased amounts of glucose-6-

phosphate and 6-phosphogluconate dehydrogenase (*61d, 61e*) as well as by the expected shift in C-6:C-1 ratio (*61f*) Since the two types of glycolysis differ in their specific requirements for DPN and TPN it is obvious why the ratio of these nucleotides has been of interest. Unfortunately careful measurements of these nucleotides in tissues of interest have sometimes, but not always been revealing, perhaps owing to compartmentalization and the presence of other systems which may interact with these nucleotides (*61g, 61h*)

In an elaborate experiment with rat liver in which the labeled sugars were supplied with perfusing blood, to minimize the confusion caused by recycling, the contribution of the oxidative pathway was deduced to be 55% (*61i*) Studies of C-6 C-1 ratios in plants have implied that the oxidative pathway plays a more important role in the older tissues than in meristimatic tissue (*61j, 61k*)

A somewhat different approach which can indicate whether ribose arises by the oxidative pathway or by the transaldolase-transketolase requires the use of specifically labeled glucose or a precursor of glucose. Afterwards, ribose (and if necessary, glucose) is isolated and degraded In an early experiment of this type labeled formate, glycine, or acetate was supplied to chcks The distribution of label in glucose, from glycogen, and in ribose, from RNA, excluded the direct formation of the ribose skeleton from the hexose skeleton (*61l*) A later interpretation showed the results to be consonant with the origin of ribose by the transaldolase-transketolase transformation (*61m*) Ribose formation from glucose, in the rat, occurred by the oxidative route to the extent of 30 to 50% and the remainder by the transaldolase-transketolase route (*61n*) In the same experiment, thiamine deficiency decreased the relative contribution of the transaldolase-transketolase sequence as would be anticipated from the known requirement of the transketolase for thiamine pyrophosphate

In general, the isotope experiments strongly support the premise that an operative pentose phosphate pathway is widely distributed However even the most refined experiments with isotopically labeled metabolites frequently yield results which are offered with reservations and are acceptable only as qualitative guides In general, results which indicate exclusive participation by the fermentative pathway are regarded as [illegible] pretation of such experiments Difficulties arise because of exchange reactions, compartmentalization, redistribution of labeled carbon by the nondehydrogenase portion of the pentose phosphate pathway and various other factors (*61m, 62*)

D The Pentose Phosphate Cycle in the Path of Carbon in Photosynthesis

The path of carbon in photosynthesis is now well understood It encompasses as an essential feature the nondehydrogenase portion of the pentose phosphate cycle Two additional reactions which act upon phosphopentoses are unique features of the pentose circuit in photosynthetic tissue One is the carboxylating enzyme which condenses carbon dioxide with ribulose diphosphate to produce 3-D-phosphoglyceric acid and the other is the phosphorylation of D-ribulose-5-phosphate to produce ribulose diphosphate The over-all path of carbon in photosynthesis is shown in Fig. 8.

1. *Diphosphoribulose Carboxylase*

This enzyme, sometimes called "carboxydismutase," is present in a photosynthetic tissue *(63–66)* and in chemosynthetic organisms It i generally but not always absent from nonphotosynthetic tissues o organisms *(67)* In photosynthetic tissue this enzyme is associated wit the chloroplasts and may be identical with the so-called "Fraction I" of Wildman and Bonner *(68)* "Fraction I" is a high molecular weight protein found in the soluble protein extracts of a variety of green leaves *(69)* It frequently comprises the major portion of the soluble protein and is, in any event, the largest single component. It can be recognized by its electrophoretic behavior and by its sedimentation

Astasia which is believed to be an albino derivative of *Euglena* contains the enzyme, although, curiously, artificially bleached *Euglena* does not *(70)* *E coli*, which is unequivocally a heterotroph, but which can fix carbon dioxide, elaborates this enzyme when it is grown on pentose i the presence of CO_2 *(71)*

2 *D-Ribulose-5-phosphate Kinase*

This enzyme which has been studied intensively in spinach leaves *(72)* has been crystallized *(73)* It is obviously present in all photosynthetic tissue It has thus far been shown to phosphorylate only D-ribulose-5-phosphate ATP is the only suitable phosphate donor. The enzyme possesses an essential sulfhydryl group and requires a divalent cation such as Mg^{++} The equilibrium conditions are overwhelmingly in favor of the diester

3. *TPN-D-glyceraldehyde-3-phosphate Dehydrogenase*

The reduction of the 3-phosphoglyceric acid, or more properly, 1,3-diphosphoglyceric acid, results from the action of TPNH rather than

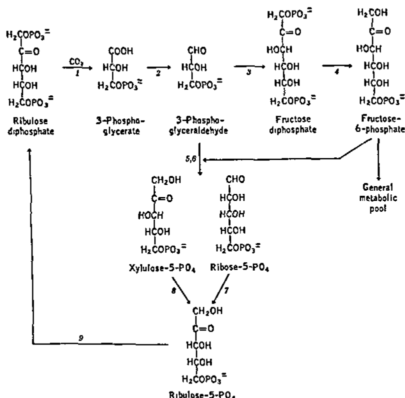


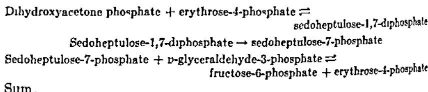
FIG 8 Path of carbon in photosynthesis The individual reactions are catalyzed by the following enzymes: *1* Diphosphoribulose carboxylase, *2* Glyceraldehyde phosphate dehydrogenase, *3* Aldolase, *4* Fructose-1,6-diphosphatase, *5*, Transketolase, *6* Transaldolase, *7* Phosphoriboisomerase, *8* D-Xylulose-5-phosphate epimerase, *9* D-Ribulose-5-phosphate kinase

DPNH as normally observed in the glycolytic sequence (*74*). In general the TPN enzyme is mainly present in photosynthetic tissues.

4 *Fructose-1,6-diphosphatase*

The cleavage of the phosphate on C-1 of fructose-1,6-diphosphate is clearly a necessary step in the carbon cycle as shown in Fig 8. Two enzymes are found in spinach leaves which may carry out this cleavage (*75*) One, operative at pH 8 5, is specific for this hydrolysis. Another, with its pH optimum at neutrality, attacks the 1-phosphate group of both fructose diphosphate and sedoheptulose-1,7-diphosphate It is

believed that this second enzyme is the effective one in the pathway because it is present in the chloroplasts while the other is not. A variation in the pathway has been suggested which would provide an additional role for this enzyme. Instead of the condensation of two triose phosphates by aldolase, it is proposed that D-erythrose-4-phosphate may condense with a triose phosphate.

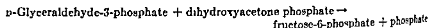


Dihydroxyacetone phosphate + erythrose-4-phosphate ⇌ sedoheptulose-1,7-diphosphate

Sedoheptulose-1,7-diphosphate → sedoheptulose-7-phosphate

Sedoheptulose-7-phosphate + D-glyceraldehyde-3-phosphate ⇌ fructose-6-phosphate + erythrose-4-phosphate

Sum.



D-Glyceraldehyde-3-phosphate + dihydroxyacetone phosphate → fructose-6-phosphate + phosphate

III. Oxidative Metabolism of Non-phosphorylated Glucose

A. Oxidation of Glucose

Enzymes which oxidize glucose directly to gluconolactone are to be found widely distributed among various species although in many instances the relative significance of this reaction to the over-all carbohydrate metabolism remains to be established. Examples of oxidases and dehydrogenases are given below.

1. *Fungal Glucose Oxidase*

"Notatin" is one of the trivial names given to the aerodehydrogenase elaborated by *Penicillium notatum*. While this is the only enzyme of its class which has been highly purified, it is presumably representative of the glucose oxidases produced by other fungal species (*76*). This enzyme (*77–80*) which utilizes O_2 directly through a FAD coenzyme and therefore produces H_2O_2 is highly specific for β-glucose and can be used as a specific reagent for glucose.

2. *Algal Glucose Oxidase*

The red alga *Iridophycus flaccidum* contains an oxidase which is effective not only on glucose but also on galactose and a variety of disaccharides (*81*). Here also the lactone is the primary oxidation product. Although H_2O_2 is produced in the reaction, the enzyme has not been sufficiently purified to permit the detection of a functional flavin nucleotide

3 *Mammalian Glucose Dehydrogenase*

Rat (*82*) or beef liver (*83*) contain an enzyme which catalyzes the dehydrogenation of glucose at the expense of DPN or TPN

4 *Bacterial Glucose Oxidase*

Pseudomonas fluorescens possesses an unusual glucose oxidase in which the oxidation by atmospheric oxygen is mediated through a cytochrome system (*84*)

B FURTHER UTILIZATION OF GLUCONOLACTONE

As in the case of 6-phosphogluconolactone, the gluconolactone ring will open spontaneously but normally one may expect a lactonase to be present in the tissue containing the oxidase or dehydrogenase. The ΔF of delactonization at pH 6 7 is appreciable (-4700 cal/mole) and makes a substantial contribution toward driving the reaction in the direction of dehydrogenation The ΔF of the dehydrogenation is only -2410 cal/mole at the same pH (*83*).

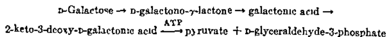
The gluconate may now undergo a variety of reactions depending on the organism and in the case of bacteria may sometimes involve induced enzymes (*40, 85a*) For instance, phosphorylation may produce 6-phosphogluconate which may then be further metabolized by pathways discussed earlier, or by the Entner-Doudoroff pathway described below. In certain special cases further oxidation may occur without further phosphorylation, resulting in 2-keto, 5-keto and 2,5-diketo derivatives A comprehensive survey of the action of a number of microorganisms is to be found in reference (*85b*)

C "ENTNER-DOUDOROFF" PATHWAY

The catabolism of glucose, fructose, and mannose by *Pseudomonas saccharophila* occurs by a novel series of reactions commonly known as the Entner-Doudoroff pathway (*86, 87*) (Fig 9)

The operation of this scheme has now been rigorously established for this organism as well as for *P fluorescens* (*88a*) The reactions are found to a limited extent in other species

Galactose is metabolized in *P saccharophila* cultures which have been adapted to this sugar (*88b*) Galactose is apparently not phosphorylated until late in the sequence



D-Galactose → D-galactono-γ-lactone → galactonic acid →

2-keto-3-deoxy-D-galactonic acid $\xrightarrow{\text{ATP}}$ pyruvate + D-glyceraldehyde-3-phosphate

Glucose	6-Phosphoglucose	6-Phosphogluconate	2-Keto-3-deoxy-6-phosphogluconate	
CHO	CHO	COO^-	COO^-	COO^- Pyruvate
HCOH	HCOH	HCOH	C=O	C=O
HOCH	HOCH	HOCH	CH_2	CH_3
HCOH →	HCOH →	HCOH $\xrightarrow{a}$	HCOH $\xrightarrow{b}$	CHO 3-Phosphoglyceraldehyde
HCOH	HCOH	HCOH	HCOH	HCOH
CH_2OH	$CH_2OPO_3^-$	$CH_2OPO_3^-$	$CH_2OPO_3^-$	$CH_2OPO_3^-$

FIG 9 The Entner-Doudoroff pathway.

IV. Pentose Metabolism in Bacteria

The pentose phosphate pathway as described above is operative in a number of bacterial species. In addition there are a number of reactions and sequences involving pentoses which are limited to a few species of bacteria and indeed are often induced by pentose alimentation Several representative examples from among the many species studied are given

A. Pentose Metabolism in *E. coli*

The utilization of free pentoses by various strains of *E coli* is dependent on their conversion to the necessary phosphorylated derivatives, which are then further metabolized by the enzymes of the pentose phosphate pathway.

D-Arabinose is a rarely encountered sugar and is not utilized by many microorganisms However, strains of *E. coli* have been isolated which are capable of metabolizing D-arabinose (*40*). This function was shown to be adaptive, and extracts of cells grown on D-arabinose catalyzed the transphosphorylation from ATP to pentose, whereas cells grown on glucose were unable to effect this reaction (*89*) Cells adapted to D-arabinose were also capable of fermenting ribulose but not ribose. Analysis of this phenomenon has shown the presence of two enzymes in these cells, the first, an adaptive pentose isomerase (*44*) catalyzing the reaction.

$$\text{D-Arabinose} \rightleftharpoons \text{D-ribulose}$$

and the second, the specific transphosphorylase, ribulokinase, catalyzing the reaction.

$$\text{Ribulose} + \text{ATP} \rightarrow \text{ribulose phosphate} + \text{ADP}$$

This pentose isomerase is the first isomerase known for the free sugars The equilibrium produces about 85% arabinose at pH 8, the pH optimum

However, the equilibrium point may be shifted toward ribulose at a higher pH, and this suggests the possible existence of an intermediary enediol, in a reaction analogous to the Lobry de Bruyn transformation investigated by Topper and Stetten (*90*) In the presence of 0 1 *M* borate at pH 8, which traps ribulose and not arabinose, the enzyme may be used to effect a 70% to 90% conversion of D-arabinose to ribulose, this is a yield many times higher than that so far obtained by any other chemical method The reaction is specific for these sugars among the pentoses and therefore can provide a specific analytical tool for these substances In addition, L-fucose, which resembles D-arabinose in carbons 1 to 5 (see Fig 10), is converted by the enzyme to a compound which gives ketose reactions

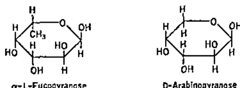


FIG 10 The configurational relationship of L-fucose and D-arabinose

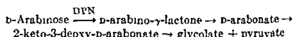
The arabinose isomerase has been separated from the transphosphorylase specific for ribulose from extracts of *E coli* It had been observed earlier that the product of D-arabinose metabolism in these extracts was an acid-stable pentose phosphate Thus the product of ribulose phosphorylation was either itself a 5-phosphate or was converted to a moiety of this type such as ribose-5-phosphate It may be noted that straight-chain sugar phosphates such as ribulose-5-phosphate (*42*) or 2-deoxy derivatives (*91*, *92*) are not as stable to acid as a compound such as the furanose, ribose-5-phosphate, which may have been the final product analyzed in the study of D-arabinose metabolism

B. PENTOSE METABOLISM IN *Pseudomonas*

The metabolism of D- and L-arabinose by this organism involves first their direct oxidation to the lactone of their respective onic acids This type of oxidation is not unknown with other microorganisms, as for example, certain bacteria of the acetic acid group have been reported to produce D-arabonic acid from D-arabinose (*93*) and indeed pentonic acids from xylose and ribose (*94*). Hayasida (*95*) isolated arabonic and xylonic acids formed by a species of *Fusarium* from arabinose and xylose, respectively

1 D-*Arabinose*

Cultures of *Pseudomonas saccharophila* adapted to arabinose cataboliz[e] this sugar by a 2–3 cleavage, which is unique in that phosphorylation i[s] not involved (*96*). The following sequence has been demonstrated fo[r] this reaction, with the carboxyl groups of the pyruvate and glycolat[e] arising from the C-1 and C-4 of arabinose, respectively.

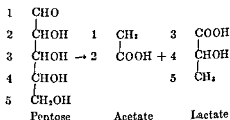


2 L-*Arabinose*

L-Arabinose is converted by *P saccharophila* to L-arabonolactone, thence to L-arabonic acid and finally to α-ketoglutaric acid, without the participation of phosphorylated intermediates (*97*).

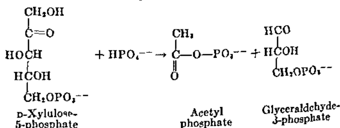
C PENTOSE METABOLISM IN *Lactobacillus plantarum*

This organism, more familiarly known as *Lactobacillus pentosus*, and related strains, quantitatively convert D-xylose, L-arabinose, and D-ribose (*98*) to a mixture of acetic and lactic acid Isotopic experiments show the carbon of the products to be derived in the following manner.



The details of the conversion of these sugars to D-xylulose-5-phosphate (*99–102*) are shown in Fig 11

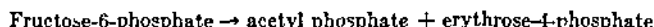
The xylulose-5-phosphate then undergoes a novel phosphorolytic cleavage, catalyzed by "ketolase," a thiamine pyrophosphate-dependent enzyme, as shown (*103*) and the products are then converted to acetate and lactate by well known sequences



CHO HCOH HCOH HCOH CH_2OH D-Ribose	Ribokinase → ATP	CHO HCOH HCOH HCOH $CH_2OPO_3^{--}$ D-Ribose-5-phosphate	Phosphoribo- ⇌ isomerase	CH_2OH C=O HCOH HCOH $CH_2OPO_3^{--}$ D-Ribulose-5-phosphate ⇅ 3-epimerase
CHO HCOH HOCH HCOH CH_2OH D-Xylose	D-Xylose isomerase ⇌	CH_2OH C=O HOCH HCOH CH_2OH D-Xylulose	D-Xylulose kinase → ATP	CH_2OH C=O HOCH HCOH $CH_2OPO_3^{--}$ D-Xylulose-5-phosphate ⇅ 4 epimerase
CHO HCOH HOCH HOCH CH_2OH L-Arabinose	L-Arabinose isomerase ⇌	CH_2OH C=O HOCH HOCH CH_2OH L-Ribulose		CH_2OH C=O HOCH HOCH $CH_2OPO_3^{--}$ L-Ribulose-5-phosphate

FIG 11 Conversion of aldopentoses to D-xylulose-5-phosphate in *Lactobacillus plantarum*

Two moles of ATP are produced for each mole of pentose which is cleaved, a highly satisfactory yield for a fermentative reaction All of the enzymes involved in the separate pathways are known In *Acetobacter xylinum*, a similar phosphoketolase occurs which acts upon fructose-6-phosphate in an analogous manner (*104*)



Fructose-6-phosphate → acetyl phosphate + erythrose-4-phosphate

D PENTOSE METABOLISM IN *Aerobacter*

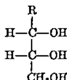
In *Aerobacter aerogenes* the conversion of L-arabinose to D-xylulose-5-phosphate occurs in exactly the same manner as shown above (*105–107*) The D-xylulose-5-phosphate is then further metabolized via the transaldolase and transketolase system and subsequently by the usual glycolytic enzymes

E FORMATION OF KETULOSES

Two naturally occurring pentitols, D-arabitol and ribitol (adonitol), (*108*) are known Both appear to be quite rare but are encountered in

plant tissues, the distribution of ribitol apparently being even more limited than that of arabitol. It would be of interest to know if these compounds arose by reduction of a ketose, as in the conversion of fructose to sorbitol

Acetobacter strains oxidize the sugar alcohols at the grouping



containing contiguous *cis*-hydroxyls D-Arabitol is converted to D-xyloketose or D-xylulose (*109*), and D-ribitol to L-riboketose or L-ribulose (*110a*) (Fig 12).

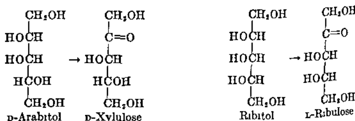


FIG 12 The fermentative conversion of the pentitols to ketopentoses

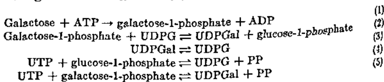
V. Uridine Diphosphoglycosides

A Galactose Metabolism

1. *Interconversion of Galactose and Glucose*

The transformation of glucose-1-phosphate and galactose-1-phosphate involves the intermediates, uridine diphosphogalactose (UDPGal) and uridine diphosphoglucose (UDPG, Fig. 13).

The enzymic steps concerned with the formation of glucose-1-phosphate from galactose are shown below:



$$\text{Galactose} + \text{ATP} \rightarrow \text{galactose-1-phosphate} + \text{ADP} \quad (1)$$
$$\text{Galactose-1-phosphate} + \text{UDPG} \rightleftharpoons \text{UDPGal} + \text{glucose-1-phosphate} \quad (2)$$
$$\text{UDPGal} \rightleftharpoons \text{UDPG} \quad (3)$$
$$\text{UTP} + \text{glucose-1-phosphate} \rightleftharpoons \text{UDPG} + \text{PP} \quad (4)$$
$$\text{UTP} + \text{galactose-1-phosphate} \rightleftharpoons \text{UDPGal} + \text{PP} \quad (5)$$

Reaction 1, catalyzed by galactokinase, is interesting because phosphorylation occurs on the aldehydic oxygen instead of the primary

alcoholic oxygen as is the case with hexokinase The enzyme, first discovered in galactose-adapted yeast (*110c*), is found in a number of organisms and especially in mammals as might be expected from the importance of lactation in these animals

The second enzyme (Eq 2), galactose-1-phosphate uridyl transferase, also found in galactose-adapted yeast (*110d*) involves UDPG. The third enzyme (Eq 3), uridine diphosphoglycosyl-4-epimerase was also found in galactose-adapted yeast (*111a*) It will be noted that UDPGal may also

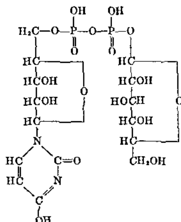


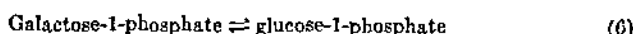
FIG 13 The structure of uridine diphosphoglucose (*110b*).

arise via uridine diphosphogalactose pyrophosphorylase (*111b*) (Eq. 5), although in mammalian liver this possibility is not considered important

The summation of Eqs (2) and (3) gives.

$$\text{Galactose-1-phosphate} \rightleftharpoons \text{glucose-1-phosphate} \qquad (6)$$

It is thus clear why UDPG is required only in catalytic amounts and [illegible]



(*111c*)

The epimerase itself (Eq 3) appears to catalyze an "oxidation-reduction-oxidation" at the C-4 position of the UDP-hexose The enzyme which has been isolated from the liver in a highly purified form shows a dependence on DPN (*112*) A similar enzyme from *Lactobacillus bulgaricus*, if it does utilize DPN, must have the coenzyme bound as an

integral part of the enzyme (*113*). No exchange of carbon-bound H is observed when the epimerization occurs in D_2O or T_2O, showing that DPNH, if it is formed, must utilize the same H in the reduction step (*113*, *114*) An intermediate carbonyl compound has not been detected

2 *Galactosemia*

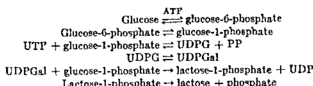
The biochemical lesion which is responsible for the appearance of the ordinary type of congenital galactosemia is the abnormally low content of galactose-1-phosphate uridyl transferase (Eq 2) in the erythrocytes (*115*) and in the liver (*116*) The enzyme deficiency results in the accumulation of high concentrations of galactose-1-phosphate in the red blood cells of afflicted subjects (*117*) This accumulation can also be observed *in vitro* (*111b*, *117*) upon the addition of galactose to the blood from galactosemics. The pathological manifestation of the disease cannot be due to an inability of the patient to obtain galactose in a biochemically useful form, for as can be seen from Eqs (2) and (3), glucose can be converted to a biochemically useful derivative of galactose The cause of the toxicity is not clear Suggestions have been made that galactose-1-phosphate may exert its effect by inhibiting phosphoglucomutase (*118*, *119a*) or that the free galactose may interfere with transmembranal transfer of glucose by inhibiting mutarotase (*119b*). Another possibility is the increased demand for ATP caused by the abnormal accumulation of phosphate esters (*117*). Galactose in the diet of galactosemics produces hypoglycemia, hence it has been suggested that the homeostatic mechanism for maintaining blood glucose may not be able to distinguish between glucose and galactose (*120a*). Fortunately, the disease is adequately controlled by withholding galactose or milk from the diet

While a continued diet of 40% galactose is toxic, the feeding of such a diet to rats does not alter the normal pattern of glucose metabolism by liver slices (*120b*)

As noted above, the action of UDP-galactose pyrophosphorylase (Eq 5) might also serve as an alternate route for the introduction of galactose-1-phosphate into the metabolic stream Small amounts of this enzyme have been found in rats, the quantity increasing with the age of the animal (*120c*)

3 *Biogenesis of Lactose*

The homogenate of the bovine udder converts UDP-galactose + glucose-1-phosphate to lactose and UDP (*121a*, *121b*) Thus, the over-all formation of lactose from glucose may be portrayed in terms of enzymes found in the udder as follows·



$$\text{Glucose} \xrightleftharpoons{\text{ATP}} \text{glucose-6-phosphate}$$
$$\text{Glucose-6-phosphate} \rightleftharpoons \text{glucose-1-phosphate}$$
$$\text{UTP} + \text{glucose-1-phosphate} \rightleftharpoons \text{UDPG} + \text{PP}$$
$$\text{UDPG} \rightleftharpoons \text{UDPGal}$$
$$\text{UDPGal} + \text{glucose-1-phosphate} \rightarrow \text{lactose-1-phosphate} + \text{UDP}$$
$$\text{Lactose-1-phosphate} \rightarrow \text{lactose} + \text{phosphate}$$

hexoses does not occur However, when glucose-C^{14} is perfused into the udder, the lactose formed is labeled in both moieties as could be expected from the action of the enzymes shown (*121c*)

The ruminant meets the major part of its carbon requirement with low molecular weight fatty acids Hence, the hexose moieties of the lactose produced by the cow are ultimately derived from these fatty acids However, when radioactive acetate is injected into the general circulation of the lactating animal, the glucose and galactose moieties of the lactose are dissimilarly labeled (*122a*) The glucose is labeled predominantly in the C-3 and C-4 positions much as one anticipates with glycogen glucose The labeling in the galactose, however, is rather significantly dispersed among C-1, C-2, C-3, and C-4 At first glance this is an unexpected result if one postulates that galactose and glucose moieties are derived from a common substance, glucose-1-phosphate The anomaly is even more disturbing when acetate-1-C^{14} or propionate-1-C^{14} is perfused with blood into the isolated udder, for now only 3 and 6%, respectively, of the activity of the lactose appears in the glucose moiety (*122b*)

If acetate is injected unilaterally into one-half of the cow's udder via the appropriate artery, the galactose moiety is asymmetrically labeled (*123*) The administration of glucose-2,6-C^{14} and glucose-6-C^{14} also results in an anomalous distribution (*124*) The explanation is advanced that the glucose moiety arises rather directly from free glucose and is, of course, subject to serious dilution with endogenous glucose Galactose is believed to have its origin in hexose phosphates which are subject to randomization via the pentose phosphate-sedoheptulose phosphate interchange as well as by the action of triosephosphate isomerase and aldolase (*124*)

A soluble system obtained from guinea pig mammary gland has been reported to form lactose by utilizing glucose-1-phosphate as the source of the galactose moiety and starch as the source of the glucose (*125*) Presumably, glycogen would replace starch *in vivo* It is, of course, possible that glycogen might supply both halves of the lactose By a

reversal of the uridine nucleotide glycogenesis (see Chapter 3), UDP-glucose could be obtained which would then be epimerized to UDP-galactose

B Uridine Diphosphouronic Acids

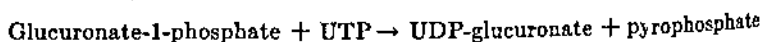
UDP-Glucuronic acid can arise by the dehydrogenation of UDP-glucose Enzymes catalyzing this transformation are found in rat liver (*126*), peas (*127*), and pneumococcus (*128*).



UDP-Glucose + 2 $DPN^+ \rightarrow$ UDP-glucuronate + $2H^+$ + 2DPNH

Although the reaction requires two moles of DPN, there is no evidence, even with a purified enzyme preparation, that the reaction is resolvable into two stages Efforts to isolate an intermediate 6-aldehydo-nucleoside have not been successful (*126*) If there are two oxidative steps, they must indeed be tightly coupled

By analogy with UDPG-phosphorylase it is to be expected that glucuronate-1-phosphate might be formed as shown.



Glucuronate-1-phosphate + UTP $\rightarrow$ UDP-glucuronate + pyrophosphate

The results were negative with the yeast and liver enzymes when tested with the α- or β-anomers (*129*) In mung beans there is an enzyme (*or enzymes*) *which catalyzes this reaction in addition* to the analogous reaction with galacturonic acid-1-phosphate (*130, 131*). Both reactions are reversible. The metabolic significance of these enzymes awaits evidence that the 1-phosphate esters of the uronic acids can be formed biologically by a kinase or by some other pathway.

C. Uridine Diphosphopentoses and Uronides in Higher Plants

Polysaccharides which contain uronic acids are well known in higher plants, e g , pectins and hemicelluloses These polyuronides are frequently accompanied by the hexoses, glucose and galactose, and by the pentoses, L-arabinose and D-xylose From the pattern of *biosynthesis* of numerous oligosaccharides and polysaccharides which is now emerging, it is entirely reasonable to propose that in the case of the plant polysaccharides the primary glycosyl donor is a uridine diphosphoglycoside This proposal is strongly supported by the discovery that the UDP-glycosides of D-glucose, D-galactose, D-xylose, L-arabinose (*132a*), D-glucuronate, and D-galacturonate occur in higher plants (*131*).

A comparison of the structure of glucuronic acid with that of D-xylose and galacturonic acid with that of L-arabinose strongly suggests that the pentoses originate by decarboxylation.

The labeling pattern in the D-galacturonic *acid* and L-arabinose derived from pectin which has been synthesized in the boysenberry (*132b*) and in the strawberry (*133*) from specifically labeled glucose is in harmony

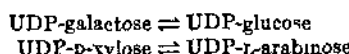
with the above formulation Extensive studies made with specifically labeled monosaccharides as well as glucuronolactone in the biosynthesis of polysaccharides in wheat have also provided substantial support for this concept (*134–136*). The remarkably high conversion of glucuronolactone to xylan in the corn coleoptile also confirms the decarboxylation hypothesis (*137*)

Finally, the hypothesis that the UDP-monosaccharides are involved in the synthesis of many polysaccharides has received excellent support from the demonstration of the following enzymic interconversions in extracts of higher plants.



UTP + sugar-1-phosphate ⇌ UDP-sugar + pyrophosphate

where the sugar may be α-D-glucose, α-D-galactose, α-D-xylose, β-L-arabinose and α-L-arabinose (*138*) Furthermore, these extracts catalyze the following epimerase reactions (*138*).



UDP-galactose ⇌ UDP-glucose
UDP-D-xylose ⇌ UDP-L-arabinose

Particulate preparations of mung beans also convert UDP-glucuronate to UDP-galacturonate These substances are converted by decarboxylation to UDP-D-xylose and UDP-L-arabinose (*139*).

D Uridine Diphosphoglycosides in the Biosynthesis of Saccharides

The discovery that UDPG acts as a glycosyl donor in the formation of sucrose and trehalose has set the stage for the discovery of a number

Table I
Saccharide Syntheses Requiring UDP Donors

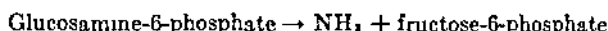
Saccharide	Donor	Organism	Reference
Cellulose	UDP-glucose	*Acetobacter*	(*140, 141*)
Chitin	UDP-acetylglucosamine	*Neurospora*	(*142, 143*)
β-1,3-Glucan	UDP-glucose	Mung bean	(*144*)
Glucosyl-D-xyluloside	UDP-glucose	Pea	(*145*)
Glucosyl-D-rhamnoside	UDP-glucose	Pea	(*145*)
Glucosyl-L-sorboside	UDP-glucose	Pea	(*145*)
Glycogen	*UDP-glucose*	Mammalian muscle	(*146*)
Glycogen	UDP-glucose	Mammalian liver	(*147*)
Hyaluronic acid	UDP-glucuronate UDP-acetylglucosamine	Rous sarcoma	(*148*)
Lactose-phosphate	UDP-galactose	Cow	(*118*)
Sucrose phosphate	UDP-glucose	Wheat germ	(*149*)
Sucrose	UDP-glucose	Wheat germ	(*150*)
Trehalose-phosphate	UDP-glucose	Yeast	(*151*)

of analogous reactions, and it is confidently to be expected that the list will grow The participation of UDP-glycosides in a variety of saccharide syntheses is summarized in Table I (For details concerning these reactions see Chapter 6.)

VI. Hexosamine Metabolism

A. Hexosamine Deaminase

Hexosamine in order to be metabolized must be first phosphorylated, a reaction which can be catalyzed by hexokinase (*152*) Enzymes for the subsequent deamination of glucosamine-6-phosphate are found in bacteria and animal tissues (*153–156*) When care is taken to exclude phosphohexoisomerase, it can be shown that deamination occurs as follows·

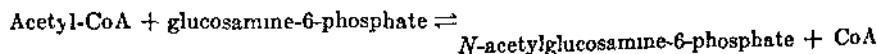


Glucosamine-6-phosphate → NH_3 + fructose-6-phosphate

For a reason which is still obscure, the presence of *N*-acetylglucosamine-6-phosphate increases the rate of deamination with deaminase preparations obtained from kidney (*154*) and *E coli* (*157*). The *N*-acetyl derivative of galactosamine-6-phosphate has a similar effect on the kidney deaminase but not on the *E coli* enzyme (*157*) Both deaminases are specific for glucosamine-6-phosphate The reaction is slightly reversible with both enzymes, but this reversibility is markedly enhanced by the presence of *N*-acetyl glucosamine in the case of the kidney enzyme Contrary to earlier beliefs (*154*), *N*-acetylglucosamine does not act as a direct participant in the reactions which it appears to accelerate The amination reaction can be driven by coupling the reaction with acetyl CoA and glucosamine phosphate acetylase (*157*).

B Acetylation of Glucosamine-6-phosphate

Enzymes which catalyze the acetylation of glucosamine-6-phosphate are widely distributed among microorganisms (*158, 159*) and animal tissue (*159*).

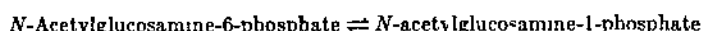


Acetyl-CoA + glucosamine-6-phosphate ⇌ *N*-acetylglucosamine-6-phosphate + CoA

Glucosamine-6-phosphate *N*-acetylase of *Neurospora crassa* has been purified (*159*) In most cases even crude extracts of the enzyme are active only on glucosamine-6-phosphate and not free glucosamine. Liver extracts contain, in addition, an acetylase which utilizes the latter compound (*159*)

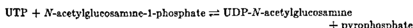
C FORMATION OF UDP-*N*-ACETYLHEXOSAMINE

The reactions leading to the conversion of *N*-acetylglucosamine-6-phosphate to the UDP derivative proceed by a course analogous to the conversion of glucose-6-phosphate to UDPG First, a phosphohexose mutase reaction must occur

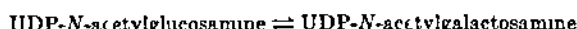

N-Acetylglucosamine-6-phosphate ⇌ *N*-acetylglucosamine-1-phosphate

Such an enzyme has been found in *Neurospora crassa* (*160*) Glucose-1,6-diphosphate serves as a coenzyme although, oddly, the enzyme functions poorly as a phosphoglucomutase Conversely, phosphoglucomutase of mammalian origin is only slightly active on *N*-acetylglucosamine phosphate

The product of reaction can now be converted to the UDP analog by a pyrophosphorylase enzyme (*161*, *162*)


UTP + *N*-acetylglucosamine-1-phosphate ⇌ UDP-*N*-acetylglucosamine + pyrophosphate

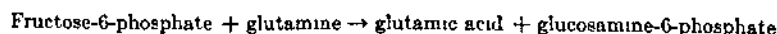
Although the direct conversion of UDP-*N*-acetylgalactosamine by an analogous pyrophosphorylase has not yet been demonstrated, this compound can arise by an epimerase reaction (*163*, *164*)


UDP-*N*-acetylglucosamine ⇌ UDP-*N*-acetylgalactosamine

The liver preparations which serve as a source of the epimerase contain a highly active hydrolase which is reported to yield *N*-acetylglucosamine on the basis of a colorimetric test of incompletely known specificity (*165*) A similar enzyme preparation has been found to yield not *N*-acetylglucosamine, but rather the corresponding mannosamine (*166*) This result implies that the primary uridine derivative is UDP-*N*-acetylmannosamine, for authentic *N*-acetylgalactosamine is not converted to *N*-acetylmannosamine It would be interesting to explore the possibility of a 2-keto intermediate In the interconversion of glucose-6-phosphate to mannose-6-phosphate, two phosphohexose-isomerases are required, which act upon a common product, fructose-6-phosphate (Chapter 3). UDP-*N*-acetylglucosamine has been found in mung bean plants (*167*)

D AMINATION OF HEXOSE PHOSPHATE

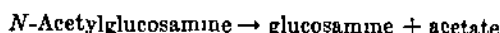
Extracts obtained from *Neurospora crassa* form glucosamine-6-phosphate according to the following reaction (*168*)


Fructose-6-phosphate + glutamine → glutamic acid + glucosamine-6-phosphate

A similar enzyme is found in rat liver When the preparation is crude, glucose-1-phosphate or glucose-6-phosphate *will replace the fructose* ester, but on purification of the enzyme, fructose-6-phosphate clearly becomes superior to the glucose ester.

E. *N*-Acetylglucosamine Deacetylase

The deacetylating enzyme can be found in a variety of bacteria (*169*).



E. coli extracts can be obtained which will hydrolyze the galactose analog, but will not attack the glucosamine derivative. No activity was found in a variety of rabbit tissues tested

VII. Neuraminic Acid Compounds

A. Structure

Although as long ago as 1936 a representative of the neuraminic acid (or sialic acid) compounds was isolated and crystallized (*170*), only

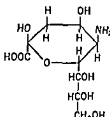


Fig 14 The structure of neuraminic acid

recently has the chemistry of these compounds been clarified and their physiological importance appreciated. The early disregard of these compounds by all but a handful of biochemists is surprising in the face of the fact that mucolipids from brain (*171*), erythrocytes (*172*), and bovine salivary mucoprotein (*173*), to cite only a few examples, contain over 20% of this material.

Neuraminic acid (Fig 14) is now, by convention, the name applied to the parent 9-carbon carbohydrate (*174*) The sialic acids are the acetylated forms occurring in various mucoproteins and mucolipids In the former, especially, they are associated with D-galactose, D-mannose, and L-fucose

N-Acetyl neuraminic acid is readily degraded by chemical means to *N*-acetyl-D-glucosamine and pyruvic acid Both *N*-acetylglucosamine and *N*-acetylmannosamine are epimerized to a mixture of these two

substances, in which N-acetylglucosamine predominates (1 explains the original claims that the hexose moiety was glucose

Enzymic degradation by a preparation obtained from *C perfringens* yields the mannose derivative (*176*) This enzyme, neuraminic aldolase catalyzes the following reversible reaction



N-Acetylneuraminic acid ⇌ pyruvic acid + N-acetyl-D-mannosar

The chemical synthesis of N-acetylneuraminic acid by the tion of N-acetylglucosamine and oxalacetate has, on the other offered to support the contention that the hexose moiety i Unfortunately, the reaction is carried out at pH 11 and hence m hydroxyl ion-catalyzed epimerization (*177*).

Isotopic feeding experiments with the rat show that the car of glucose is directly incorporated into the hexose (*178*)

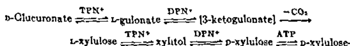
B NEURAMINIDASE

Special interest attaches to neuraminic acid derivatives the specificity of influenza virus for sialic acid end groups of mu (*179–181*) The ability of the virus to cause agglutination of er is a consequence of the presence of sialoproteins on the surf cells The virus acts as a true enzyme in causing this aggluti can be released to repeat this reaction with fresh cells N-Ac minic acid is released in this reaction The enzyme, neuramin act on other sialoproteins (*182*) and will also attack such sir compounds as N-acetylneuraminolactose, an O-glycoside of lac in milk (*113*) or a simple neuraminic acid disaccharide obta bovine salivary gland mucoprotein (*183*).

VIII. Metabolism of Non-phosphorylated Uronic Aci

A GLUCURONATE-XYLULOSE PATHWAY

The entry of glucuronic acid into the ordinary glycolytic can occur through a sequence of reactions culminating in D- phosphate



D-Glucuronate $\xrightleftharpoons{TPN^+}$ L-gulonate $\xrightleftharpoons{DPN^+}$ [3-ketogulonate] $\xrightarrow{-CO_2}$

L-xylulose $\xrightleftharpoons{TPN^+}$ xylitol $\xrightleftharpoons{DPN^+}$ D-xylulose $\xrightleftharpoons{ATP}$ D-xylulose-

The conversion of glucuronate to L-xylulose in pentosu result of an impaired physiological operation of this pathway The decarboxylation (*186*) of glucuronate and the formation

(*187*, *188*) has been demonstrated with tissue extracts The *in vitro* reduction of glucuronate and dehydrogenation of L-gulonate is well established in mammalian tissues (*185–189*) The conversion of xylitol-C^{14} *in vivo* to $C^{14}O_2$ and to glycogen in the rat and guinea pig further support the biological significance of this scheme (*190*)

The individual enzymic steps are thoroughly demonstrated although the mechanism of the oxidative decarboxylation of L-gulonate is still obscure This reaction may be a branch point in the metabolism of gulonate controlling its conversion to either xylulose or ascorbic acid. It is noteworthy that the formation of the particular enantiomorph by the dehydrogenation of xylitol is under the control of a specific pyridine nucleotide (*191*) The enzyme forming L-xylulose is highly specific but the other enzyme attacks many polyols reminiscent of mammalian "iditol dehydrogenase" The enzyme which converts D-xylulose to the 5-phospho

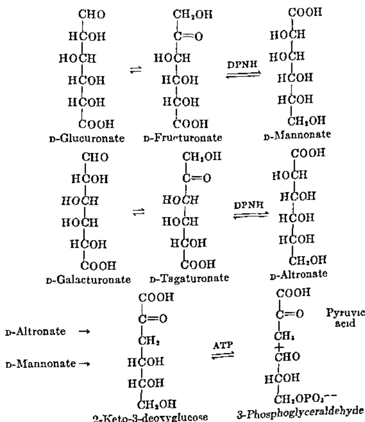


FIG 15 Ketouronate metabolism in *Escherichia coli*

derivative and thus enables it to enter the pentose phosphate pathway is also present in mammalian tissue (*192*). A bacterial D-xylulose kinase has been previously mentioned.

The curious occurrence of some L-arabitol in the urine of a pentosuric receiving D-glucuronolactone-1-C^{14} has been noted (*193*)

B KETOURONIC ACIDS

Escherichia coli grown on D-galacturonic acid as the sole source of carbon elaborate a soluble enzyme system which attacks both D-galacturonic and D-glucuronic acids as shown in Fig 15 (*194, 195*).

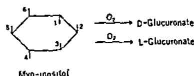
IX. Inositol Metabolism

A BIOSYNTHESIS OF INOSITOL

Yeast supplied with specifically labeled glucose, acetate, or formate synthesizes inositol whose labeling pattern indicates that it cannot be formed by cyclization of the intact glucose chain (*196*) The cyclization theory had been attractive because of the similarity of the stereochemical structure of the 4-carbon segment of glucose (C-2–C-5) with that of inositol

B CATABOLISM OF INOSITOL

The breakdown of inositol, which seems to occur predominantly in the kidney, begins with a remarkable oxidative cleavage of the ring to yield a racemic mixture of glucuronic acid (*197*)

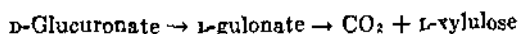


The D-isomer would be expected to arise by C-1–C-6 cleavage. When inositol-2-C^{14} is used, the D-glucuronate is indeed labeled in C-5 The enzyme which forms D-glucuronate can be obtained free of the original mixture which produces the racemate (*198*). Although the fate of the L-glucuronate is not known, it is reasonable to suppose that D-glucuronate, *in vivo*, would be transformed by the pathway previously discussed (*199–201*)

In confirmation of this proposal, administration of inositol-2-C^{14} to rats results in glycogen in which the glucose is labeled equally in the C-1

and C-6 positions (*199*) This is the anticipated consequence of the subsequent formation of D-xylulose-5-phosphate, and the transformation of the latter to hexose phosphate via the pentose phosphate pathway. In addition, it is necessary to postulate that an extensive exchange occurs between the triose phosphates derived from both halves of the hexose The glucuronate isolated from the urine is labeled differently from the glycogen glucose, having 70% more radioactivity in C-6 than in C-1. Essentially similar experiments with inositol-2-D yielded essentially glucose-6-D (*201*), but in this latter experiment one must reckon with the possibility that D on C-1 may become labilized and exchanged with H from H_2O.

The possibility that inositol catabolism may proceed through an additional pathway is suggested by the following study (*202*). Rat kidney contains the inositol → glucuronate transforming enzyme referred to above, in soluble form. The crude soluble extract converts a portion of uniformly labeled inositol-C^{14} to $C^{14}O_2$ as expected on the basis of the known reaction sequence:

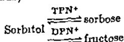

D-Glucuronate → L-gulonate → CO_2 + L-xylulose

In the presence of an excess of unlabeled D-glucuronate or L-gulonate the rate of $C^{14}O_2$ output is greatly suppressed, as anticipated A similar enzyme preparation containing added mitochondria also produces $C^{14}O_2$, but neither D-glucuronate nor L-gulonate seriously alters the rate of $C^{14}O_2$ production This suggests the suppression of the first system and the operation of an alternate system.

X. Hexitol Metabolism

In the seminal vesicles of sheep there are two enzymes which act on sorbitol One, previously described, is a DPN-dehydrogenase which forms fructose The other is a TPN-dehydrogenase which forms glucose (*203*) This arrangement provides a point of metabolic control which makes the TPN/DPN ratio a determining factor in the diversion of sorbitol to glucose or fructose.

A somewhat analogous situation arises in sorbitol-grown *Acetobacter suboxydans* (*204*, *205*). Thus,

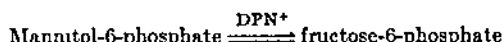


Still another sorbitol dehydrogenase is found in this organism, which by

contrast to the other enzymes is particulate-bound and shows no nucleotide requirement (*206*)

Polyol dehydrogenase continues to be found throughout the bacterial world, often being inducible, as for example, iditol (*207*) and galactitol dehydrogenase (*208*). Frequently these possess broad specificities

A polyol dehydrogenase of special interest is mannitol-6-phosphate dehydrogenase of pneumococcus (*209*)

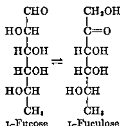


This is the first demonstration of the elaboration of an enzyme brought about by a transforming principle It is significant that the transforming principle does not actually cause the appearance of the enzyme, but rather permits it to be induced by mannitol

XI. Metabolism of Deoxyaldoses

A Fucose Metabolism

As predicted earlier (*44*), the ketose formed from L-fucose by *E coli* cultures which have been adapted to D-arabinose is indeed L-fuculose (*210*)

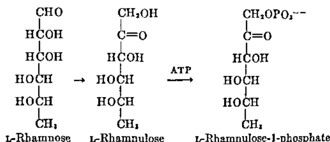


The enzyme appears to be identical with the D-arabinose isomerase. It also catalyzes the epimerization of L-galactose and D-altrose The equilibrium ratio results in an 11% conversion of fucose, the presence of borate increases this value to 85% A similar enzyme can be induced in *E freundii* L-Fuculose kinase is also formed by sequential induction (*211*).

A strain of *Aerobacter* produces an extracellular polysaccharide which contains L-fucose as one of the constituent sugars The fucose is formed directly from the carbon chain of glucose, judging from the fact that glucose-1-C^{14} and glucose-6-C^{14} are converted by the organism to fucose-1-C^{14} and fucose-6-C^{14}, respectively (*212*, *213*).

B. Rhamnose Metabolism

The isomerase and the kinase, whose actions are shown below, are induced in *E coli* cultures grown on rhamnose (*214, 215*).

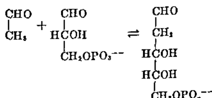


Isotopic experiments indicate the hexose skeleton of fructose is transformable directly to L-rhamnose in *Pseudomonas aeruginosa* When α,α'-C^{14}-labeled glycerol is the carbon source, the rhamnose formed is labeled in the 1,2,5,6 positions (*216, 217*)

C Deoxyribose Metabolism

Although 2-deoxyribose-5-phosphate can be synthesized enzymically from triose phosphate and acetaldehyde, it is not regarded as likely that this is the route to the deoxyribose of DNA Incorporation of totally labeled cytidine into both DNA and RNA of the rat occurs without cleavage of the nucleoside The specific activity, on a carbon basis, of the cytosine is equal to that of the pentose Hence, it is clear that the transformation of the ribose to deoxyribose can occur while the glycosidic linkage is retained (*218*) Similar experiments with nucleotides support this view (*219*).

The discovery in *E coli* and in rat liver of a deoxyribose aldolase (*220*) suggested that deoxyribose might arise *in vivo* from acetaldehyde and D-glyceraldehyde phosphate.



However, *Lactobacillus plantarum* cultures adapt to deoxyribose fermentation by elaborating both the deoxyribose aldolase and deoxyribokinase. This suggests that the aldolase is concerned primarily with

catabolism rather than the formation of deoxyribose The products of fermentation are ethanol, acetic acid, acetaldehyde, and lactic acid If bisulfite is present, stoichiometric quantities of acetaldehyde are formed (*221*). The extracts of cells so adapted can be used to degrade ribose and deoxyribose obtained from the respective nucleic acids in tracer experiments The labeling pattern is consistent with the view that the ribose chain is converted directly to deoxyribose (*222*)

XII. Carbohydrate Metabolism in Insects

The comparative biochemistry of carbohydrate metabolism reveals both similarities and differences in insects as compared to higher animals Thus, the normal glycolytic and oxidative pentose pathways are present (*223*, *224*) Trehalose may play a major role as a reserve carbohydrate being an important component of the hemolymph of a large number of insects (*225*) As discussed elsewhere (Chapter 3), glyceraldehyde phosphate dehydrogenase and α-glycerophosphate oxidase may be of unique value in insect muscle in taking over the function of lactic acid dehydrogenase and "pyruvic acid oxidase" in mammalian muscle

The hemolymph of the silkworm contains polyol dehydrogenase(s) which utilizes $TPNH^+$ in the reduction of a number of aldehydes such as glyoxal, glyceraldehyde, galacturonic acid, glucuronic acid, ribose-5-phosphate and other substances, but not hexoses and pentoses (*226*)

At the onset of diapause in the silkworm larva, the glycogen content drops markedly, but returns to normal after diapause is broken During this interval most of the glycogen is converted to sorbitol and glycerol which disappear when the glycogen returns (*227*)

ACKNOWLEDGMENTS

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CHAPTER 6

Biosynthesis of Complex Saccharides

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This chapter will be concerned chiefly with the mechanisms involved in the synthesis and degradation of complex saccharides from monosaccharides But it will begin with a brief outline of the present state of knowledge of the

Carbohydrates are generally considered to be the main ultimate products of carbon dioxide transformation in the plant by the photosynthetic process. The monosaccharides, which are produced in a series of stepwise reactions, appear to serve two major functions: they are partially consumed in respiration with the production of energy, which is utilized for the numerous metabolic reactions of the plant, they also serve as building units in being combined by various enzymic systems to form complex saccharides, such as sucrose, maltose, starch, and cellulose.

I. Photosynthetic Intermediates Leading to the Formation of Monosaccharides

Smith (*1*) showed that when photosynthesis is allowed to proceed in the sunflower for 30 minutes or longer, more than 95% of the reduced carbon dioxide can be accounted for as carbohydrate. It therefore appears that the intermediates between carbon dioxide and carbohydrate do not accumulate to any considerable extent, and that their concentration must be low by comparison with that of the final products

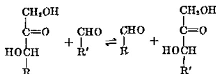
Calvin and Benson (*2*), working with *Chlorella* and *Scenedesmus*, showed that during very short photosynthetic periods (2 seconds) approximately 90% of the carbon was fixed in 3-phosphoglyceric acid This tricarbon compound appeared to be the first stable intermediate in the photosynthetic transformation of carbon dioxide to sugars Because of the known relationship between the hexose phosphates and phosphoglyceric acid in the glycolytic sequence, they assumed that the hexose phosphates are formed from phosphoglyceric acid by a mechanism similar to the reversal of glycolysis. When barley shoots were allowed to photosynthesize in radioactive carbon dioxide for 15 seconds and the distribution of activity in the 3-carbon atoms of phosphoglyceric acid was compared with that in the hexose derived from the hexose phosphate, the results were as follows (*3*). The hexose possessed the highest radioactivity in carbon atoms 3 and 4 (52%), and considerably less activity in carbon atoms 2 and 5 (25%) and 1 and 6 (21%) The carboxyl carbon of the glyceric acid contained 49% of the activity, whereas the carbon with the secondary hydroxyl and that with the primary hydroxyl contained 25% and 26% of the activity, respectively. It thus appears that the hexose is formed by the combination of two 3-carbon molecules derived from the glyceric acid in such a way that carbon atoms 3 and 4 of the hexose correspond to the carboxyl-carbon of the glyceric acid; carbon atoms 2 and 5 to the α-carbon; and carbon atoms 1 and 6 with the β-carbon of the glyceric acid. A similar correspondence in activity

between the carbon atoms of these two compounds, glyceric acid and hexose, was maintained when compared during periods of from 2 to 60 seconds

More recently Gibbs and Kandler (*4*) and Gibbs and Cynkin (*5*) found that the distribution of C^{14} activity in the D-glucose of sucrose, D-glucose phosphate, and starch formed from $C^{14}O_2$ during short-time photosynthesis in *Chlorella* and the leaves of higher plants was asymmetric Initially the carbon atom 4 was more active than the 3, and later there was more activity in 1 and 2 than in 5 and 6 This asymmetric labeling is not clearly understood at present

Examination of the products formed in the first seconds of photosynthesis by Calvin (*6*) and Bassham and Calvin (*7*) showed that besides phosphoglyceric acid, other phosphorylated sugars, including D-sedoheptulose 7-phosphate, D-ribulose 5-phosphate, D-glucose 6-phosphate, and D-fructose 6-phosphate, are produced

The formation of D-sedoheptulose 7-phosphate was demonstrated by Horecker and Smyrniotis (*8*) and Horecker *et al* (*9*) as a result of the action of an enzyme, transketolase, on pentose phosphate Transketolase has been shown to split D-xylulose 5-phosphate (it was first thought that D-ribulose 5-phosphate is a substrate) to triose phosphate and an "active glycolaldehyde" This active aldehyde readily reacts with a number of suitable aldehyde acceptors, such as glycolaldehyde, D-glyceraldehyde, D-glyceraldehyde 3-phosphate, and D-ribose 5-phosphate, to form the corresponding keto sugars



Active transketolase preparations were obtained from spinach by Horecker and Smyrniotis (*10*), and this enzyme was crystallized by Racker *et al* (*11*) In addition to D-xylulose 5-phosphate, D-sedoheptulose 7-phosphate and D-fructose 6-phosphate can serve as 2-carbon ketol donors

The D-sedoheptulose 7-phosphate in the presence of D-glyceraldehyde 3-phosphate can be cleaved by another enzyme, transaldolase (*12*) in such a manner that the dihydroxyacetone is transferred to triose phosphate The transaldolase, which is present in yeast and animal and plant tissues, is specific for D-sedoheptulose 7-phosphate and D-fructose 6-phosphate as donors of the dihydroxyacetone group, and for D-glyceraldehyde 3-phosphate, D-erythrose 4-phosphate, and D-ribose 5-phosphate as

acceptors. The interconversion of sugar phosphates produced as a result of transketolase and transaldolase activity is shown by the reactions in Fig. 1.

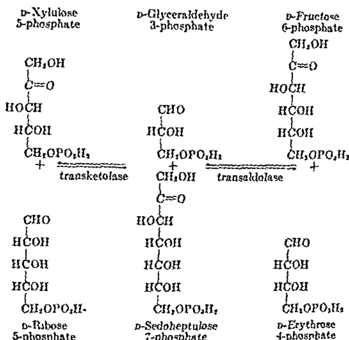


FIG. 1. Interconversion of sugar phosphates as a result of transketolase and transaldolase activities.

D-Ribulose 5-phosphate is a product of phosphogluconic decarboxylation. By the action of the widely distributed enzyme, D-ribose 5-phosphate isomerase (13, 14), D-ribulose 5-phosphate is converted to D-ribose 5-phosphate. D-Ribulose 5-phosphate is also isomerized to D-xylulose 5-phosphate by an enzyme, D-xylulose 5-phosphate isomerase, found in animal and plant tissues and in microorganisms (15–17). In plants, D-ribulose 5-phosphate is converted to D-ribulose 1,5-diphosphate through phosphorylation by adenosine triphosphate in a reaction catalyzed by the enzyme "phosphoribulokinase" (18, 19).

The carboxylation reaction by which phosphoglycerate is formed from CO_2 and some constantly generated acceptor molecule has been a matter of intense interest. Calvin and his collaborators (20–22) formerly assumed that the glyceric acid is synthesized by the addition of radioactive CO_2 to some preexisting 2-carbon fragment in the plant, possibly glycolic acid. However, recent investigations indicate that the CO_2

acceptor is a 5-carbon compound rather than a 2-carbon fragment The resultant 6-carbon molecule then gives rise to two molecules of phosphoglyceric acid

Calvin (*6*, *23*) presented evidence, based on the observation that carboxylation occurs in a cell-free system, that D-ribulose 1,5-diphosphate is the primary CO_2 acceptor in photosynthesis Weissbach *et al.* (*24*) and Mayaudon *et al* (*25*) also showed that cell-free extracts obtained from *Chlorella* contain an enzyme (or enzymes) capable of catalyzing the carboxylation of D-ribulose 1,5-diphosphate, specifically, to form phosphoglyceric acid Degradation of this acid obtained from inactive D-ribulose diphosphate and $C^{14}O_2$ in the presence of the *Chlorella* extracts showed that it contained all the radioactivity in the carboxyl carbon

In considering D-ribulose 1,5-diphosphate as a possible CO_2 acceptor for the formation of phosphoglyceric acid Calvin *et al* (*6*, *7*, *24*, *25*) postulated the mechanism shown in Fig 2

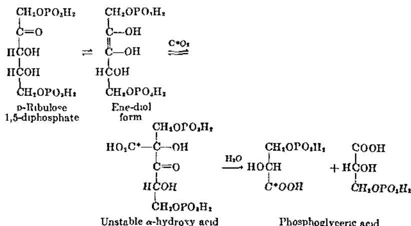
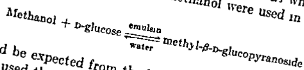


FIG 2 Mechanism of the carboxylation reaction

Calvin (*6*, *23*) suggested that this reaction proceeds via a 6-carbon keto acid diphosphate intermediate, 2-carboxy-3-ketopentenol 1,5-diphosphate. Later Moses and Calvin (*26*) succeeded in isolating from *Chlorella* two photosynthetic phosphorylated products, 2-carboxy-4-ketopentitol and the lactone of 2-carboxy-3-ketopentitol The latter compound is thought to be the result of photosynthetic carboxylation of D-ribulose 1,5-diphosphate and to give rise to 3-glycerophosphoric acid, but the role of the 2-carboxy-4-ketopentitol is not yet known

II. Reversible Reactions Catalyzed by Hydrolytic Enzymes

Synthesis of disaccharides from monosaccharides by hydrolytic enzymes was reported early in this century. Hill (42), subjecting a 40% glucose solution to the action of a yeast extract containing α-glucosidase, showed that a small amount of maltose, together with another disaccharide, was found. Similar results were reported by Fischer and Armstrong (43). Bourquelot (44) and Bourquelot and Bridel (45) demonstrated that the same rotational equilibrium was obtained when methyl-β-D-glucopyranoside was mixed with water and emulsin as when the corresponding amounts of D-glucose and methanol were used in the presence of the same enzyme.



$$\text{Methanol} + \text{D-glucose} \underset{\text{water}}{\overset{\text{emulsin}}{\rightleftharpoons}} \text{methyl-}\beta\text{-D-glucopyranoside}$$

As would be expected from the law of mass action, a large excess of methanol caused the point of equilibrium to shift toward the formation of methyl glucopyranoside. By application of this principle, using the appropriate alcohols, aliphatic and cyclic, β-D-glucopyranosides were prepared. A similar series of α-D-glucopyranosides was formed when α-glucosidase instead of emulsin was used. Gentiobiose (46) could also be obtained when a bitter almond extract (β-glucosidase) was allowed to act on D-glucose. Inasmuch as it was necessary to use concentrated sugar solutions for the preparation of these saccharides in order to force the reaction backward, it is evident that these conditions are highly artificial from the biological point of view.

Generally, complex saccharides are almost completely degraded by hydrolytic enzymes. For example, when invertase is allowed to act upon a dilute solution of sucrose, such as exists in plant cells, the reaction in which invert sugar is formed goes almost to completion. Theoretically, when equilibrium is reached, a finite amount of sucrose might remain in solution, its concentration being determined by the free energy change of the reaction and the concentrations of the hydrolysis products. Since the ΔF for the hydrolysis of sucrose is approximately -6500 cal per mole, the reaction has a strong tendency to go to the right. This tendency is greatly increased by the fact that one of the reactants is water and its concentration is enormously increased because the reaction takes place in aqueous medium. These factors are responsible for the fact that the reaction of sucrose hydrolysis is practically irreversible.

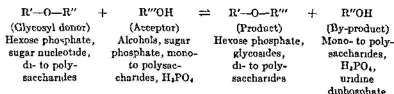
Using sensitive chromatographic techniques, it has been observed (47–49) that yeast invertase forms small amounts of di- and trisaccharide during the early stages of its action on sucrose. Mold invertase forms

longer chains, up to six or seven units, but no polysaccharide To explain the formation of these oligosaccharides, it has been suggested that invertase, in addition to being a hydrolytic enzyme, is also a fructose-transferring enzyme The oligosaccharides are formed by transfructosylation as a result of competition of the decomposition products of the substrate for the elements of water These oligosaccharides are eventually hydrolyzed in the latter stages of the reaction (*49*, *50*) Many of the previously described "hydrolytic" enzymes (lactases, maltases, cellobiases, etc) appear to be similar in this respect, and it has been suggested (*51*, *52*) that all hydrolytic enzymes capable of attacking more than one substrate may act as transferases The hydrolytic enzymes do not appear to be of direct importance in the synthesis of sucrose in higher plants, lactose in milk, starch, and many other complex carbohydrates in nature

III. Mechanisms of Oligosaccharide Formation

A Synthesis of Sucrose and Analogs of Sucrose by Sucrose Phosphorylases

The general process for the synthesis of complex saccharides (oligosaccharides, glycosides, and polysaccharides) is that of transglycosylation In this process the glycosyl donor may be sugar phosphate, sugar nucleotide, oligosaccharide, or polysaccharide The reaction may be expressed by the equation



R′—O—R″	+	R‴OH	⇌	R′—O—R‴	+	R″OH
(Glycosyl donor) Hexose phosphate, sugar nucleotide, di- to polysaccharides		(Acceptor) Alcohols, sugar phosphate, mono- to polysaccharides, H_3PO_4		(Product) Hexose phosphate, glycosides, di- to polysaccharides		(By-product) Mono- to polysaccharides, H_3PO_4, uridine diphosphate

Certain species of bacteria, namely, *Pseudomonas saccharophila*, *Pseudomonas putrifaciens*, and *Leuconostoc mesenteroides* (*53*), contain a phosphorylase which, in the presence of inorganic phosphate, catalyzes the phosphorolytic decomposition of the disaccharide, sucrose, with the production of α-D-glucose 1-phosphate and D-fructose The reverse reaction, the dephosphorolytic condensation of α-D-glucose 1-phosphate and D-fructose, results in the formation of sucrose with the elimination of phosphoric acid, as shown in Fig 4

The synthetic process can be regarded as a condensation reaction in which the elements of water of hydrolysis are replaced by those of phosphoric acid The process can also be considered as a transglucosylation

reaction in which the glucosyl radical from the α-D-glucose 1-phosphate is transferred to a fructofuranoside radical, serving as an acceptor.

The free energy required for the formation of the glycosidic link in sucrose is available in the α-D-glucose 1-phosphate and is transferred along with the D-glucose part of the ester to D-fructose. Phosphorylated D-glucose is required because free D-glucose, on account of its low free-energy level, cannot serve as part of the substrate for sucrose synthesis The energy level of the D-glucose can be raised through combination with phosphate by using the energy drop from adenosine triphosphate

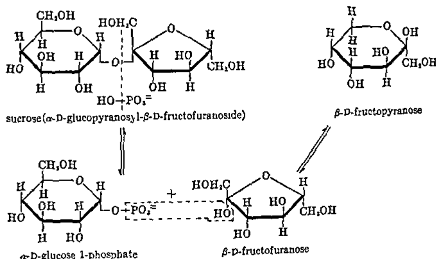


Fig 4 Phosphorolysis of sucrose

(ATP) to form D-glucose 6-phosphate through the hexokinase reaction. The latter ester can then readily be transformed by the aid of phosphoglucomutase to α-D-glucose 1-phosphate. It therefore appears that the free energy for the synthesis of sucrose is ultimately derived from ATP.

The fact that sucrose can be synthesized from α-D-glucose 1-phosphate and D-fructose is in accord with the evidence brought forward by Isbell and Pigman (*54*) and by Gottschalk (*55*) that D-fructose exists as an equilibrium mixture of the pyranose and furanose forms The total system of sucrose synthesis can be represented by the equation (Fig 4), which includes the equilibrium reaction of the two ring forms of D-fructose The fact that D-fructose occurs in the sucrose molecule as fructofuranose indicates that sucrose phosphorylase is specific to the furanose configuration of that ketose.

In the phosphorolysis reaction of sucrose the rupture of the bond in α-D-glucose 1-phosphate could occur either between the carbon and

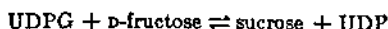
oxygen of the hexose phosphate or between the oxygen and phosphorus Cohn (*56*), employing O^{18}-labeled inorganic phosphate, has determined the location of the bond that is broken in the reaction Incubating oxygen-labeled inorganic phosphate with sucrose in the presence of sucrose phosphorylase, and allowing the reaction to proceed to equilibrium, she demonstrated that the O^{18} concentration of the inorganic phosphate and of the α-D-glucose 1-phosphate, after equilibrium had been reached, was the same as the O^{18} concentration of the initial inorganic phosphate. This could occur only if the forward and reverse reactions do not involve a rupture of the bond between phosphorus and oxygen

At equilibrium the catalyzed reaction favors the breakdown rather than the synthesis of sucrose The equilibrium constant of the reaction at pH 6 6 and 30° expressed by the mass-law equation.

$$K = \frac{[\text{sucrose}][\text{phosphate}]}{[\text{fructose}][\text{glucose 1-phosphate}]}$$

is approximately 0 05 and increases slightly at lower pH values From the equilibrium constant the free-energy change for the phosphorolysis reaction is calculated to be 1770 cal Assuming that the ΔF of the reaction is entirely due to the difference in bond energies of sucrose and α-D-glucose 1-phosphate, and taking the energy value of the C—O—P bond of the ester as 4800 cal, the bond energy of sucrose is estimated to be 6570 cal This relatively high value for the glycosidic bond in sucrose may account for the distinctive role of this disaccharide in the metabolism of the plant.

Although the presence of sucrose phosphorylase in some microorganisms provides an enzymic mechanism for the formation of sucrose, from α-D-glucose and D-fructose, this pathway does not seem to be significant in sucrose synthesis by higher plants It appears that uridine diphosphate D-glucose is involved as a D-glucose donor in the reaction (*57–61*) Cardini *et al* (*57*) showed that wheat germ, corn germ, bean germ, and potato sprouts contain an enzyme that catalyzes the reversible formation of sucrose from uridine diphosphate D-glucose (UDPG) and D-fructose

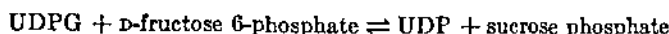


$$\text{UDPG} + \text{D-fructose} \rightleftharpoons \text{sucrose} + \text{UDP}$$

When a mixture of UDPG and D-fructose is subjected to the action of enzyme preparations from these plant sources in the presence of inorganic phosphate buffer at pH 7 0, a nonreducing disaccharide, identified as sucrose, is formed. The equilibrium constant, K, for this reaction at 37° was found to be between 2 and 8, and the ΔF was estimated as approximately -1000 cal as compared to $+1770$ cal for the sucrose phosphorylase reaction. In contrast to the sucrose phosphorylase reaction,

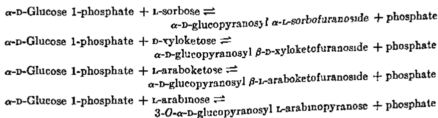
the equilibrium of the reaction starting with UDPG and D-fructose is in favor of sucrose synthesis

Leloir and Cardini (58) found later that wheat germ contains another enzyme which will form sucrose phosphate when D-fructose 6-phosphate is substituted for D-fructose.


UDPG + D-fructose 6-phosphate ⇌ UDP + sucrose phosphate

Bean and Hassid (62) showed that the same enzyme preparation from green peas, in addition to synthesis of sucrose from UDPG and D-fructose, and *sucrose phosphate from the same nucleotide and D-fructose 6-phosphate*, is capable of forming other disaccharides (sucrose analogs) when ketose monosaccharides other than D-fructose or D-fructose 6-phosphate are used as D-glucose acceptors Thus, when D-rhamnulose, D-xylulose, or L-sorbose was used in the presence of this pea preparation and UDPG, D-glucosyl D-rhamnuloside, D-glucosyl D-xyluloside, or D-glucosyl L-sorboside was formed, respectively The latter two disaccharides are probably identical with those formed by the action of sucrose phosphorylase, an enzyme present in *Pseudomonas saccharophila*, from D-glucose 1-phosphate and the corresponding ketoses (63).

Sucrose phosphorylase is capable of synthesizing analogs of sucrose in which the D-fructose is replaced by other ketose sugars (53). D-Xyloketose, L-araboketose, and L-sorbose can replace D-fructose in the reaction with α-D-glucose 1-phosphate, forming the corresponding nonreducing disaccharides, D-glucosyl D-xyloketoside, D-glucosyl L-araboketoside, and D-glucosyl L-sorboside Inasmuch as it has been shown that these disaccharides are nonreducing and that their ketose constituents exist in the furanose form, they are considered as analogs of sucrose The sucrose phosphorylase is also capable of combining α-D-glucose 1-phosphate with an aldose, L-arabinose, to form a reducing disaccharide with a 1,3-glucosidic linkage, having no obvious structural relation to sucrose Since most of the known natural reducing disaccharides, such as maltose, lactose, and cellobiose, possess a 1,4-linkage, this 1,3-linkage appears to be unique among disaccharides (63) Sucrose phosphorylase can catalyze the synthesis and decomposition of the following disaccharides.


α-D-Glucose 1-phosphate + L-sorbose ⇌ α-D-glucopyranosyl α-L-sorbofuranoside + phosphate

α-D-Glucose 1-phosphate + D-xyloketose ⇌ α-D-glucopyranosyl β-D-xyloketofuranoside + phosphate

α-D-Glucose 1-phosphate + L-araboketose ⇌ α-D-glucopyranosyl β-L-araboketofuranoside + phosphate

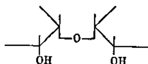
α-D-Glucose 1-phosphate + L-arabinose ⇌ 3-*O*-α-D-glucopyranosyl L-arabinopyranose + phosphate

The *Pseudomonas saccharophila* phosphorylase is highly specific with regard to the D-glucose portion of its substrate The enzyme is incapable of combining D-fructose with either α-D-galactose 1-phosphate, α-D-mannose 1-phosphate, α-D-xylose 1-phosphate, α-maltose 1-phosphate, or α-L-glucose 1-phosphate (*53*) A change in configuration of any of the hydroxyls in the α-D-glucopyranosyl radical appears to prevent its combination with the enzyme and inasmuch as an enzyme-substrate complex is not formed, the glucopyranosyl radical cannot be transferred to the D-fructose acceptor

The ability of the enzyme to combine α-D-glucose 1-phosphate with a number of monosaccharides other than D-fructose shows that the enzyme is far less specific with regard to substituents for the second half of the sucrose molecule, D-fructose

Besides inorganic phosphate, several keto-monosaccharides, namely L-sorbose, D-xyloketose, L-araboketose, and the aldopentose, L-arabinose, can serve as D-glucose acceptors in the transfer reaction

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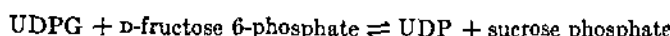
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B Synthesis of Disaccharides by Transglucosylation

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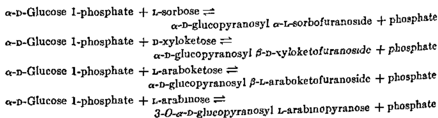
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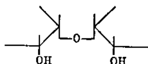
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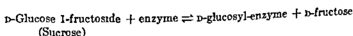
"transglucosylase" capable of mediating the transfer of the D-glucose portion of substrates to a variety of "acceptors" *(65)* The evidence for the double function of the enzyme was adduced from the observation that when P^{32}-labeled inorganic phosphate and nonradioactive α-D-glucose 1-phosphate were added to sucrose phosphorylase preparations in the absence of ketose sugars, a rapid redistribution of the isotope occurred between the organic and inorganic fractions without the liberation of D-glucose. D-Glucose, which is known to inhibit sucrose phosphorylase, was also found to inhibit the exchange reaction Similarly, the presence of D-fructose was found to decrease the rate of exchange. Such a decrease in rate would be expected if D-fructose competed with phosphate for the D-glucose residue of α-D-glucose 1-phosphate These observations led to the assumption that the enzyme combines reversibly with the D-glucose portion of α-D-glucose 1-phosphate, forming a D-glucose-enzyme complex and releasing inorganic phosphate, in accordance with the equation



α-D-Glucose 1-phosphate + enzyme ⇌ D-glucosyl-enzyme + P

The equilibrium of the reaction would require that the energy of the α-D-glucose 1-phosphate linkage be preserved in the D-glucosyl-enzyme bond The transfer of phosphate could not involve the formation of free D-glucose, because if this occurred, approximately 4800 cal would be released in the decomposition of the ester and would be required for its resynthesis Since no external source of energy was available for the resynthesis of the ester, it must be concluded that the original bond energy is conserved in the D-glucosyl-enzyme complex

It was also demonstrated *(66)* that, in a phosphate-free medium, sucrose phosphorylase brings about the exchange of added free C^{14}-labeled fructose, forming sucrose in which its D-fructose moiety proved to be radioactive

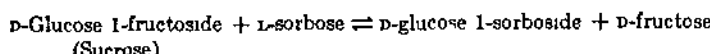


D-Glucose 1-fructoside + enzyme ⇌ D-glucosyl-enzyme + D-fructose
(Sucrose)

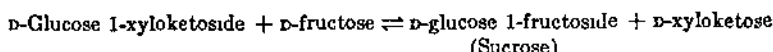
It will be observed that in this, as well as in the phosphorolysis reaction, the enzyme acts as D-glucose donor and D-glucose acceptor to its substrates, and is able to catalyze the exchange of an ester bond for a glycosidic bond The role of sucrose phosphorylase in the reversible phosphorolysis of sucrose can thus be considered as a summation of the two reactions

The transglucosylase function of this enzyme can further be demonstrated by the fact that the enzyme will catalyze an exchange of glucosidic bonds between two different disaccharides in the absence of inorganic

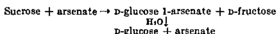
phosphate and α-D-glucose 1-phosphate Thus, D-glucosyl L-sorboside, which had been originally synthesized from α-D-glucose 1-phosphate and L-sorbose, can also be prepared by a reaction between sucrose and L-sorbose:


D-Glucose 1-fructoside + L-sorbose ⇌ D-glucose 1-sorboside + D-fructose
(Sucrose)

In a similar manner, sucrose can be prepared by a reaction between the synthetic disaccharide, D-glucosyl D-xyloketoside and D-fructose (*53*):

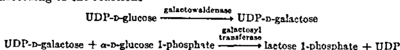

D-Glucose 1-xyloketoside + D-fructose ⇌ D-glucose 1-fructoside + D-xyloketose
(Sucrose)

The mode of action of sucrose phosphorylase explains the observed role of arsenate in causing a hydrolytic decomposition of both sucrose and α-D-glucose 1-phosphate in the presence of the enzyme (*67*). Arsenate presumably acts as a D-glucose acceptor with the enzyme, to form an unstable glucose 1-arsenate compound, which hydrolyzes spontaneously to D-glucose and arsenate:


Sucrose + arsenate → D-glucose 1-arsenate + D-fructose
H_2O↓
D-glucose + arsenate

Trehalose phosphate, known to exist in yeast, has been synthesized by Cabib and Leloir (*68*) from UDP-D-glucose and α-D-glucose to phosphate by a transglycosylase present in brewer's yeast The enzyme which catalyzes the reaction, UDP-D-glucose + D-glucose 6-phosphate → UDP + trehalose phosphate, has a maximal activity at pH 6 6 in the presence of 2 5 × 10^{-2} *M* magnesium ion The equilibrium of the reaction is displaced toward the synthesis of trehalose phosphate, the reversibility of the reaction could not be demonstrated The yeast also contains a specific phosphatase for trehalose phosphate Free trehalose is found in some higher plants and fungi It is probably formed *in vivo* from the phosphorylated precursor by hydrolysis with phosphatase

Gander *et al* (*69, 69a*), using enzyme preparations from mammary tissue of lactating cows, claim to have accomplished the synthesis of lactose 1-phosphate from UDP-D-glucose and α-D-glucose 1-phosphate according to the reactions


UDP-D-glucose —galactowaldenase→ UDP-D-galactose
UDP-D-galactose + α-D-glucose 1-phosphate —galactosyl transferase→ lactose 1-phosphate + UDP

However, there appears to be a discrepancy in properties between their biosynthetic lactose 1-phosphate and the lactose 1-phosphate prepared by chemical methods (*70*) According to Gander *et al.*, heating of

the biosynthetic compound with 1 *N* hydrochloric acid at 100° fo 10 minutes completely removed the phosphate group without affectin the lactose Contrary to this finding, it is well known that treatment of lactose 1-phosphate with 1 *N* hydrochloric acid under these conditions not only causes liberation of the phosphate, but also, to a considerable extent, will hydrolyze the lactose to its monosaccharide constituents. An attempt by R J Reithel (*70a*) to repeat Gander and co-worker's biosynthesis of lactose 1-phosphate was unsuccessful In view of these facts, the biosynthesis of lactose 1-phosphate cannot at present be considered as having been accomplished

A number of other oligosaccharides have been synthesized by th transglycosylation process by the action of various microorganisms A reducing disaccharide, 5-*O*-α-D-glucopyranosyl D-fructopyranose, named leucrose (*71*), together with another disaccharide, 6-*O*-α-D-glucopyranosyl D-fructofuranose, is formed in the reaction mixture during the synthesis of dextran from sucrose by an enzyme isolated from *Leuconostoc mesenteroides*

Aspergillus niger (strain 152), which produces an intracellular polyglucan (*72*), contains a transglucosylase responsible for the synthesis of a number of oligosaccharides When maltose is used as a substrate in the presence of a cell-wall extract of this mold, the following oligosaccharides are produced isomaltose (6-*O*-α-D-glucopyranosyl D-glucose), panose (*O*-α-D-glucopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 4)-α-D-glucopyranose), and isomaltotriose This indicates that the mold contains an intracellular transglucosylase analogous to the extracellular enzymes produced by *A niger* NRRL-337 (*73*) and by *A oryzae* (*74*).

Another trisaccharide formed from sucrose by *A niger* has been characterized as *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside (*75*)

During the action of an enzyme extract from yeasts on lactose two disaccharides and two trisaccharides are produced (*76*). 6-*O*-D-galactosyl D-glucose, 6-*O*-D-galactosyl D-galactose, *O*-D-galactosyl-(1 → 6)-*O*-D-galactosyl-(1 → 4)-D-glucose, and *O*-D-galactosyl-(1 → 6)-*O*-D-galactosyl-(1 → 6)-D-glucose

A trisaccharide, *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(6 → 2)-β-D-fructofuranoside, named kestose, is produced during the action of yeast invertase on a 50% sucrose solution (*77*). This trisaccharide is similar in structure to the one shown by Dedonder to be present in Jerusalem artichoke (*78*)

Wallenfels and Brent (*79*), using chromatographic methods, also demonstrated the existence of a transgalactosylase capable of forming D-galactose- and D-glucose-containing oligosaccharides from lactose.

By means of levansucrase-catalyzed transfer of the D-fructose group from sucrose to the anomeric carbon of lactose, Avigad (83) synthesized a nonreducing trisaccharide, "lactsucrose." This trisaccharide could be decomposed by yeast invertase to equimolar amounts of D-fructose and lactose, and by β-galactosidase to equimolar amounts of D-galactose and sucrose Structurally, the trisaccharide was shown to be *O*-β-D-galactopyranosyl-(1 → 4)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside.

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IV. Synthesis of α-Linked Polysaccharides by the Action of Phosphorylase

Because of their great biological importance, starch and glycogen have been the subject of intensive investigation for many years. It is, therefore, no accident that there should be more information available at present concerning their mechanisms of synthesis and degradation than about any other natural polysaccharide

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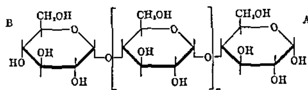


FIG 5 Amylose (*A*, reducing end, *B*, nonreducing end)

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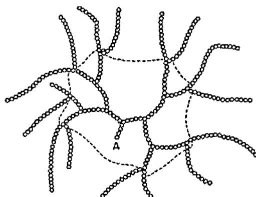


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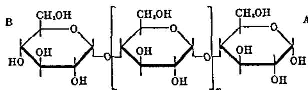


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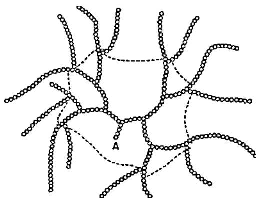


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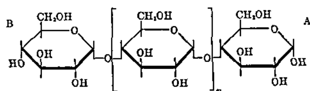


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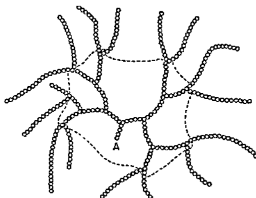


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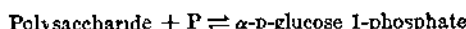
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B. Effect of pH on the Equilibrium Reaction

The equilibrium of the phosphorolysis reaction is largely influenced by the hydrogen ion concentration This may be explained by the fact that the inorganic phosphate and the ester phosphate have different acid strengths, the latter being stronger The hydrogen ion, therefore, enters into the true equilibrium equation



Under conditions of greater acidity, a larger proportion of starch and inorganic phosphate is formed Thus, when the pH value is varied from 5 0 to 7 0, the values of the ratio of inorganic phosphate to ester phosphate decrease progressively from 10 8 to 3 1 (*99*)

A similar hydrogen ion concentration effect is observed with animal phosphorylase and glycogen (*100*) The ratio of concentration of inorganic phosphate to α-D-glucose 1-phosphate, reached from either side, is 5 7 at pH 6 0 and 2 7 at pH 7 6 Although the ratio of inorganic phosphate to total α-D-glucose 1-phosphate at equilibrium varies considerably with pH, Hanes (*91*) found that the ratio of the divalent ions, $(HPO_4)^{--}$ to $(C_6H_{11}O_5OPO_3)^{--}$, remains approximately constant at a value of 2 2 over this pH range, indicating that the equilibrium is determined by the divalent ions only

C The Role of Primer

Synthesis of polysaccharides from α-D-glucose 1-phosphate by phosphorylase does not ensue unless a small amount of starch, glycogen, or dextrin is added as priming agent Cori and his associates (*101*) explained the role of the added polysaccharide as an activator by showing that it is actually a participant in the reaction The primer is required because the enzyme is unable to cause a direct condensation of α-D-glucose 1-phosphate units, but acts as a medium for transferring D-glucose units from α-D-glucose 1-phosphate to the end of an already existing chain The "priming" efficiency of a polysaccharide is a function of the number of nonaldehydic terminal D-glucose units This is evident from the fact that branched-chain polysaccharides, such as amylopectin or glycogen, having numerous nonreducing end groups, are good activators, whereas the straight-chain amylose, with only one nonreducing end group, has little effect The mechanism now generally accepted for the synthesis of amylose from α-D-glucose 1-phosphate is that advanced by Cori and collaborators (*101*) It is based chiefly on the role played by nonreducing end groups in the priming reactions If $(C_6H_{10}O_5)_x$ represents the primer,

and in bacteria, namely *Neisseria perflava* (*94*) and *Polytomella coeca* (*95*) Although the properties of phosphorylases obtained from various sources differ in certain respects (*90*), they possess the chief common characteristic of being capable of disrupting or synthesizing 1,4-α-glucosidic linkages at the nonreducing end of the glycogen or starch chain The process is reversible, and can be illustrated, as shown in Fig 7 Interaction of inorganic phosphate with the terminal glucosidic bond results in the formation of α-D-glucose 1-phosphate and a loss of one D-glucose unit in

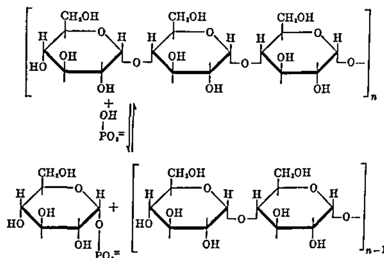


FIG 7 Phosphorolysis of amylose (according to C F Cori and G T Cori)

the chain In the reverse reaction, D-glucose units from α-D-glucose 1-phosphate are added one at a time, with the simultaneous liberation of phosphate, thus lengthening the polysaccharide chain

The reaction catalyzed by phosphorylase is in extremely mobile equilibrium which can be readily approached from both directions As in the case of sucrose phosphorylase, the rupture of the bond in the phosphorylation reaction catalyzed by muscle or potato phosphorylase occurs between the carbon and oxygen of D-glucose 1-phosphate and not between the oxygen and phosphorus (*56*).

The reversible enzymic polymerization takes place with little change in energy, as may be calculated from the equilibrium constant. The energy of the C-O-P linkages of α-D-glucose 1-phosphate (4800 cal) is approximately the same as that of the glucosidic linkages of the polysaccharides, indicating that the $\Delta F°$ for the phosphorolytic reaction must be small (*96–98*).

Weibull and Tiselius (*104*) and others (*105–107*) found that maltotriose is the smallest molecule which will act as primer for potato phosphorylase, but this is very inefficient by comparison with maltotetraose and larger maltodextrins Comparing the effectiveness of amylose and amylopectin as primers on an end-group concentration basis, Swanson and Cori (*108*) found the Michaelis constant of 3 × 10^{-4} *M* of end groups per liter for amylopectin and soluble starch, and 1 4 × 10^{-4} *M* for linear amylopentaose They concluded that the constant for corn amylose

TABLE I
MICHAELIS CONSTANTS FOR VARIOUS PRIMERS

Primer	Average chain length*	K_m (moles/liter end groups) × 10^6
Dextrin	7	17
Dextrin	23	5
Corn amylose	100	3 6
Sago amylose	220	0 9
Easter lily amylose	640	0 8
Tapioca amylose	650	0 9
Potato amylose	1450	0 4
Corn amylopectin	26	10
Apple amylopectin	24	7
Dog glycogen	15	71

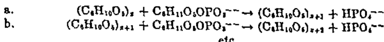
* Average chain length for the amyloses = $\frac{\text{D P measured by osmotic pressure}}{\text{number of nonreducing end-groups}}$ as determined by Potter and Hassid (*109*)

(thought to be 200 units long) was approximately the same as for amylopectin In studying further the effectiveness of various polysaccharide primers on potato phosphorylase, Neufeld (*103*) showed that while *amylopectin appears to be a more effective* primer than amylose, when comparison is made on equal weight basis, amylose proves to be more effective when the end-group concentration is taken into consideration.

Determination of the Michaelis constants of several branched polysaccharides and of a number of amyloses of different chain length calculated on the basis of end-group concentration (*109*) showed long-chain amyloses to be more effective than amylopectin and short-chain dextrins, and glycogen to be least effective in their role as primers (see Table I)

Peat *et al* (*110*) showed that potatoes contain an enzyme, named D-enzyme, capable of catalyzing the reversible disproportionation of maltodextrins, the D-glucose oligosaccharides containing the α-1,4-linkage For example, when a preparation containing D-enzyme acts on maltotriose, D-glucose and maltopentaose are produced as the first

each step in the synthesis is visualized as follows:



a. $(C_6H_{10}O_5)_x + C_6H_{11}O_5OPO_3^{--} \rightarrow (C_6H_{10}O_5)_{x+1} + HPO_4^{--}$

b. $(C_6H_{10}O_5)_{x+1} + C_6H_{11}O_5OPO_3^{--} \rightarrow (C_6H_{10}O_5)_{x+2} + HPO_4^{--}$

etc

The function of primers is to serve as receptors for D-glucose residues, which become attached stepwise at the nonreducing ends. In this way the primer chains are lengthened by repetition of the above process until their length becomes a limiting factor

The mass-law equation (a) or (b) appears to be in perfect agreement with Hanes' (*91*) observation that the equilibrium of the phosphorolysis reaction is not affected by the concentration of the polysaccharide, provided a certain minimum concentration is exceeded

$$K = \frac{[(C_6H_{10}O_5)]_{x+1}[\text{phosphate}]}{[(C_6H_{10}O_5)]_x[\text{glucose 1-phosphate}]}$$

Since each of the polysaccharide concentrations in the mass law expression represents the concentration of the nonreducing termini of the chains, the number of which does not change, these terms cancel out. It follows that at a given pH the equilibrium constant, K, will be determined entirely by the relative concentrations of phosphate and α-D-glucose 1-phosphate

It is interesting to note the exceedingly high speed of the phosphorylase reaction Cori *et al* (*102*) have calculated that under optimum conditions a mole of the enzyme transforms 4×10^4 moles of α-D-glucose 1-phosphate per minute

The synthetic product formed by the action of potato phosphorylase or crystalline muscle phosphorylase on α-D-glucose 1-phosphate, using glycogen or amylopectin as primer, produces a sparingly water-soluble product which gives a blue color with iodine and shows a chain length of more than 100 D-glucose units by end-group analysis These properties are associated with a linear configuration of natural amylose However, it was not clear whether the synthetic product constitutes a very large molecule formed as a result of addition of D-glucose units to the terminal branches of the primer, thus lengthening the chains many-fold, or whether the synthetic product consists of single linear molecules resulting from detachment of the branched primer when its growing chains exceed a certain maximum length (*101*).

An investigation of this problem by Neufeld (*103*) revealed that when amylopectin is used as primer, a branched polysaccharide of a much greater molecular weight is formed as a result of lengthening the outer branches, whereas the use of a linear primer results in the production of a linear polysaccharide, only of a longer chain length.

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products of the reaction, and at equilibrium D-glucose and a whole series of maltodextrins are present. However, with maltotriose as the initial substrate, none of the synthetic oligosaccharides is of sufficient length to give a color with iodine (the minimum chain length required for color formation is 12 D-glucose units).

Walker and Whelan (*111*) found that when yeast hexokinase and adenosine triphosphate were added to an incubation mixture containing the short-chain dextrins, an iodine-staining product was obtained which appeared to be amylose. In the presence of hexokinase and adenosine triphosphate the D-glucose is converted to D-glucose 6-phosphate, to which D-enzyme cannot transfer chain segments, thus tending to lengthen the dextrin chain and resulting in the production of amylose.

It has been suggested (*112*) that the potato may use both phosphorylase and D-enzyme to synthesize amylose, the product from phosphorylase remaining as amylose and the product from D-enzyme being converted into amylopectin by Q-enzyme.

D. Specificity of Phosphorylase

Phosphorylase is specific with regard to its action upon α-D-glucose 1-phosphate, the β-isomer cannot serve as a substrate for this enzyme. Neither can any other sugar ester be substituted for α-D-glucose 1-phosphate. α-L-Glucose 1-phosphate, maltose 1-phosphate, α-D-xylose 1-phosphate could not be polymerized to polysaccharide by potato phosphorylase (*113, 114*). The α-forms of D-mannose 1-phosphate and D-galactose 1-phosphate were not acted upon by muscle phosphorylase (*115*).

E. Phosphorolysis and Arsenolysis of Linear and Branched Polysaccharides

The conversion of an unbranched linear 1,4-α-linked polysaccharide (amylose) can be carried practically to completion if the polysaccharide is treated with phosphorylase in the presence of a sufficiently large excess of inorganic phosphate to insure that the equilibrium ratio of the bivalent ions is not attained before all the polysaccharide is degraded (*116–118*). On the other hand, the branched polysaccharide (amylopectin) is degraded by phosphorylase only to the extent of approximately 55% (*116*). This is explained by the assumption that the phosphorylase, which acts by removing successive D-glucose units at the nonreducing chain ends, cannot break or bypass the 1,6-linkages at the branch points. The action of the enzyme ceases when the outer chains of the main branches of the polysaccharide have been shortened to 3 to 6 units (*119–121*).

Arsenate can replace phosphate in the degradation of these polysac-

charides by phosphorylase (*116*) However, the α-D-glucose 1-arsenate that is formed is unstable and is immediately hydrolyzed to D-glucose and arsenate This accounts for the fact that no arsenate-glucose 1-arsenate equilibrium can be established when arsenate is used The arsenolysis reaction results in the complete degradation of linear polysaccharide containing only 1,4-α-linkages, and in partial degradation of branched polysaccharide possessing 1,6-linkages in addition to 1,4-linkages

V. Synthesis of Branched Polysaccharides

A SYNTHESIS OF GLYCOGEN WITH A BRANCHING ENZYME

Polysaccharides synthesized *in vitro* by crystalline muscle phosphorylase or by potato phosphorylase resemble the amylose fraction of potato starch in that the product consists of linear molecules possessing only 1,4-glycosidic linkages (*122–125*) Since naturally occurring glycogen and starches contain 1,6-linkages in addition to 1,4-, the action of the

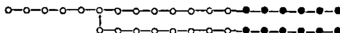


FIG 8 Segment of glycogen showing two branches, solid circles represent C^{14}-labeled glucose units

phosphorylases *in vivo*, whose function is the establishment of 1,4-linkages, must obviously be supplemented by another enzyme-catalyzed reaction through which branching is induced It is assumed that in the process of preparation of the muscle or potato phosphorylase the enzyme responsible for the formation of the 1,6-linkages is eliminated, which accounts for the *in vitro* production of the amylose-type polysaccharides Evidence for the existence of such a supplementary enzyme was first provided by Cori and Cori (*126*) They showed that several animal

formation of a polysaccharide which closely resembles glycogen

On purification of the branching enzyme from liver, a preparation was obtained having very little amylase and no phosphorylase activity. Larner (*127*) used this preparation and C^{14}-labeled D-glucose to investigate the mechanism of the transglucosidase action of this enzyme He first degraded the outer chains of glycogen to about 30% by the action of phosphorylase, and then rebuilt them to approximately their original size by means of the same enzyme and C^{14}-labeled α-D-glucose 1-phosphate, as illustrated in Fig 8

products of the reaction, and at equilibrium D-glucose and a whole series of maltodextrins are present. However, with maltotriose as the initial substrate, *none of the synthetic oligosaccharides* is of sufficient length to give a color with iodine (the minimum chain length required for color formation is 12 D-glucose units).

Walker and Whelan (*111*) found that when yeast hexokinase and adenosine triphosphate were added to an incubation mixture containing the short-chain dextrins, an iodine-staining product was obtained which appeared to be amylose. In the presence of hexokinase and adenosine triphosphate the D-glucose is converted to D-glucose 6-phosphate, to which D-enzyme cannot transfer chain segments, thus tending to lengthen the dextrin chain and resulting in the production of amylose.

It has been suggested (*112*) that the potato may use both phosphorylase and D-enzyme to synthesize amylose, the product from phosphorylase remaining as amylose and the product from D-enzyme being converted into amylopectin by Q-enzyme

D. Specificity of Phosphorylase

Phosphorylase is specific with regard to its action upon α-D-glucose 1-phosphate, the β-isomer cannot serve as a substrate for this enzyme Neither can any other sugar ester be substituted for α-D-glucose 1-phosphate. α-L-Glucose 1-phosphate, maltose 1-phosphate, α-D-xylose 1-phosphate could not be polymerized to polysaccharide by potato phosphorylase (*113, 114*) The α-forms of D-mannose 1-phosphate and D-galactose 1-phosphate were not acted upon by muscle phosphorylase (*115*).

E Phosphorolysis and Arsenolysis of Linear and Branched Polysaccharides

The conversion of an unbranched linear 1,4-α-linked polysaccharide (amylose) can be carried practically to completion if the polysaccharide is treated with phosphorylase in the presence of a sufficiently large excess of inorganic phosphate to insure that the equilibrium ratio of the bivalent ions is not attained before all the polysaccharide is degraded (*116–118*). On the other hand, the branched polysaccharide (amylopectin) is degraded by phosphorylase only to the extent of approximately 55% (*116*) This is explained by the assumption that the phosphorylase, which acts by removing successive D-glucose units at the nonreducing chain ends, cannot break or bypass the 1,6-linkages at the branch points The action of the enzyme ceases when the outer chains of the main branches of the polysaccharide have been shortened to 3 to 6 units (*119–121*)

Arsenate can replace phosphate in the degradation of these polysac-

charides by phosphorylase (*116*) However, the α-D-glucose 1-arsenate that is formed is unstable and is immediately hydrolyzed to D-glucose and arsenate This accounts for the fact that no arsenate-glucose 1-arsenate equilibrium can be established when arsenate is used The arsenolysis reaction results in the complete degradation of linear polysaccharide containing only 1,4-α-linkages, and in partial degradation of branched polysaccharide possessing 1,6-linkages in addition to 1,4-linkages

V. Synthesis of Branched Polysaccharides

A Synthesis of Glycogen with a Branching Enzyme

Polysaccharides synthesized *in vitro* by crystalline muscle phosphorylase or by potato phosphorylase resemble the amylose fraction of potato starch in that the product consists of linear molecules possessing only 1,4-glycosidic linkages (*122–125*) Since naturally occurring glycogen and starches contain 1,6-linkages in addition to 1,4-, the action of the

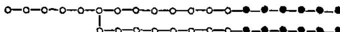


Fig 8 Segment of glycogen showing two branches, solid circles represent C^{14}-labeled glucose units

phosphorylases *in vivo*, whose function is the establishment of 1,4-linkages, must obviously be supplemented by another enzyme-catalyzed reaction through which branching is induced It is assumed that in the process of preparation of the muscle or potato phosphorylase the enzyme responsible for the formation of the 1,6-linkages is eliminated, which accounts for the *in vitro* production of the amylose-type polysaccharides Evidence for the existence of such a supplementary enzyme was first provided by Cori and Cori (*126*) They showed that several animal organs, such as the heart, the brain, and the liver, contain a "branching factor" capable of synthesizing 1,6-linkages The combined action of the branching enzyme and crystalline muscle phosphorylase resulted in the formation of a polysaccharide which closely resembles glycogen

On purification of the branching enzyme from liver, a preparation was obtained having very little amylase and no phosphorylase activity. Larner (*127*) used this preparation and C^{14}-labeled D-glucose to investigate the mechanism of the transglucosidase action of this enzyme He first degraded the outer chains of glycogen to about 30% by the action of phosphorylase, and then rebuilt them to approximately their original size by means of the same enzyme and C^{14}-labeled α-D-glucose 1-phosphate, as illustrated in Fig 8.

The re-formed glycogen was then isolated and incubated with the purified transglucosylase. If the action of the branching enzyme consisted in the shifting of chain segments by transglucosylation, the new structure would be as shown in Fig 9. This would mean that *whereas all the* C^{14}-labeled D-glucose units were previously joined in 1,4-positions, some would be combined through 1,6-linkages after branching occurred. When the latter linkages were split off as free D-glucose by means of amylo-1,6-glucosidase (debranching enzyme), the D-glucose was found to be radioactive, indicating that branching occurred within the labeled portions

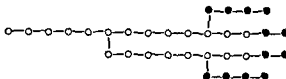


FIG 9 Formation of additional branching points in the two glycogen chains after action of branching enzyme

of the straight chains. It is likely that glycogen synthesis takes place *in vivo* through the simultaneous activity of phosphorylase and branching enzyme, and that a repetition of the process illustrated in Fig 9 would result in a multibranched structure.

Recently Leloir and Cardini (*39*) obtained a preparation from liver that produces glycogen from UDP-D-glucose. Villar-Palasi and Larner (*40*) confirmed this observation with skeletal muscle and diaphragm tissues They showed that these tissues contain two enzymes, UDP-D-glucose pyrophosphorylase and a transglycosylase which converts UDP-D-glucose to glycogen

(1) Uridine triphosphate (UTP) + α-D-glucose 1-phosphate ⇌ uridine diphosphate D-glucose + pyrophosphate (PP)

(2) UDP-D-glucose (UDPG) + glycogen primer ⇌ glycogen + uridine diphosphate (UDP)

The equilibrium of reaction (2) was found to be displaced toward glycogen synthesis

Robbins *et al* (*41*) similarly demonstrated that in pigeon breast muscle homogenate, rabbit and rat skeletal muscle, an enzyme was found that synthesizes glycogen from UDPG. These authors point out the advantages of glycogen synthesis from UDPG rather than from α-D-glucose 1-phosphate Calculations based on data for the sucrose-synthesizing enzyme, sucrose phosphorylase, and phosphorylase show that the transfer of a D-glucose unit from UDPG to glycogen should have a

standard free-energy change of about −3,000 cal. Conversion to glycogen, therefore, should be greater than 99%. In confirmation, transfers of this order of magnitude have already been observed

B SYNTHESIS OF AMYLOPECTIN WITH Q-ENZYME

Soon after Cori and Cori (*126*) showed the existence of a branching factor, Haworth *et al* (*128*) reported the isolation from potato juice of an enzyme fraction termed Q-enzyme, which, in association with potato phosphorylase, produced a polysaccharide having the properties of amylopectin Peat (*129*) and his collaborators also presented evidence that the Q-enzyme was capable of converting linear amylose to branched amylopectin without the participation of inorganic phosphate in the reaction They therefore concluded that the Q-enzyme is a nonphosphorolytic enzyme Besides being present in the potato, Q-enzyme has also been found in the wrinkled pea (*101*), the broad bean (*92*), *Neisseria perflava* (*94*), and *Polytomella coeca* (*130*)

Nussenbaum and Hassid (*131*), and Cori and Illingworth (*132*) submitted further evidence that the polysaccharide produced from amylose by the Q-enzyme is branched Like amylopectin, the product gave a purple color with iodine, and on treatment with β-amylase was hydrolyzed to maltose to the extent of 50%. Phosphorolysis with crystalline muscle phosphorylase degraded the polysaccharide to 33% α-D-glucose 1-phosphate End-group analysis by periodate oxidation shows an average of 21 D-glucose residues per end group, whereas the biological end-group assay, using amylo-1,6-glucosidase, gave an average chain length of 20 D-glucose units Estimation of the molecular weight by osmotic pressure measurements indicates a value of 54,000, which is considerably less than that of natural amylopectin Thus the synthetic product appears to be a branched polysaccharide possessing the major chemical and biochemical properties of amylopectin

In contrast to the amylolysis reaction of the amylosaccharides with the amylases, the conversion of amylose to amylopectin entails the liberation of little or no reducing groups (*131*, *133*). The Q-enzyme appears to be capable of converting about one in every twenty 1,4-linkages of the amylose into 1,6-linkages, forming a branched structure. Like the enzyme present in *Pseudomonas saccharophila* and several other microorganisms (*53*), Q-enzyme can be regarded as belonging to the class of transglycosylases

It has been demonstrated (*134*, *135*) that in the conversion of amylose to amylopectin, the linear dextrin must reach a certain size before the Q-enzyme is able to act on it. The dextrin must contain at least 42 D-glucose units before it can be attacked by the enzyme

In studying the question of the reversibility of Q-enzyme, Barker *et al* (*136*) were unable to find any conclusive evidence that the enzyme can rupture 1,6-linkages of amylopectin or β-dextrin (residual dextrin left after hydrolysis of amylopectin or glycogen with β-amylase). It is clear, however, that the equilibrium of the reaction strongly favors the synthesis rather than the scission of the branch points

When the reaction of the Q-enzyme with amylose is arrested after short periods of incubation, the synthetic polysaccharides appear to have structures intermediate between those of linear amylose and branched amylopectin (*134*). This indicates that during the course of conversion of amylose to amylopectin the action of the Q-enzyme on the amylose molecules must be random that is, the enzyme does not attack one amylose chain at a time but acts in random fashion by transferring the degraded amylose fragments to a number of other amylose molecules, forming branched structures. By varying the relative concentrations of potato phosphorylase and the Q-enzyme with α-D-glucose 1-phosphate as substrate, Barker *et al* (*137*) also obtained fractions which appeared to have a degree of branching intermediate between that of linear amylose and branched amylopectin.

Although the Q-enzyme differs from Cori's "branching enzyme" in that the latter is devoid of action toward amylose, the two enzymes possess a common property: they both act as transglucosylases capable of establishing 1,6-linkages

The claim of Bernfeld and Meutémédian (*138, 139*) that they isolated an enzyme, "isophosphorylase," capable of synthesizing 1,6-glucosidic linkages from α-D-glucose 1-phosphate in a manner similar to that by which phosphorylase establishes 1,4-linkages, could not be substantiated Bailey and Whelan (*140*) showed that their experimental data can be adequately interpreted without having to postulate the existence of an "isophosphorylase" Neither Nussenbaum and Hassid (*131*) nor Meyer (see ref. *141*) were able to repeat the preparation of "isophosphorylase"

C. Debranching Enzymes

Recently, enzymes have been discovered which are capable of degrading 1,6-α-glucosidic linkages in glycogen and amylopectin. Cori and Larner (*142*) showed that two enzymes, muscle phosphorylase and *amylo-1,6-glucosidase, are required for the complete* degradation of the branched polysaccharides, glycogen, and amylopectin The phosphorylase starts its degrading action at the nonreducing ends by breaking the 1,4-α-linkages of the outer branches of the polysaccharide in the presence of inorganic phosphate by the process of phosphorolysis, forming α-D-glucose 1-phosphate When the enzyme approaches a branch point

its action stops, because phosphorylase can neither break nor bypass a 1,6-α-linkage The exposed D-glucose units with linkages in the remaining limit dextrin are split off by the second enzyme, amylo-1,6-glucosidase, as free D-glucose, thereby opening the way for further phosphorylase action Several repetitions of this process by the combined action of the two enzymes cause the degradation of the whole branched polysaccharide molecule The ratio of free D-glucose to total D-glucose (free + phosphorylated) obtained by the action of phosphorylase plus amylo-1,6-glucosidase is characteristic of the type of branched polysaccharide being degraded, and can serve as a basis for a method of enzymic end-group determination (*143*)

Another debrancing enzyme, R-enzyme, bearing a resemblance to Cori's amylo-1,6-glucosidase, was shown to be present by Hobson *et al* (*144*) in the bean and potato This enzyme likewise hydrolyzes 1,6-linkages in amylopectin but has no action on the 1,4-linkages of either amylopectin or amylose The R-enzyme does not synthesize 1,6- or 1,4-linkages, its action is purely hydrolytic A similar debranching enzyme has also been reported in yeast by Maruo and Kobayashi (*145*) and by Petrova in muscle (*146*)

VI. Structure of Glycogen and Amylopectin

It was definitely established about two decades ago that both glycogen and amylopectin possess a branched structure However, for a number of years there has been considerable uncertainty as to the manner in which the branches are combined to form the polysaccharide molecule The English school led by Haworth and Hirst (*147*, *148*) considered each branch (consisting of approximately 25 D-glucose residues in amylopectin and 12 or 18 residues in glycogen) to be united to the next by a glycosidic linkage, operating from the potentially reducing end of the chain and jointed to the neighboring chain through one of the available sixth hydroxyl groups, thus forming a "laminated" structure, as shown in Fig 10 On the other hand, Meyer (*88*, *149*, *150*) visualized these polysaccharides as multibranched or tree-like structures, as in Fig 6.

While the multibranched model for glycogen and amylopectin has been accepted by the majority of carbohydrate chemists as the one representing a closer approximation to the true structure, the Haworth and Hirst laminated model has been seriously considered as a possibility, inasmuch as it is consistent with practically all the data upon which Meyer and Bernfeld base their multibranched structure. Most of the experimental results could be interpreted so as to fit either model (*151*, *152*) By submitting glycogen and amylopectin to separate and consecu-

tive action of phosphorylase and amylo-1,6-glucosidase, Larner *et al.* (*163*) were able to examine the inner structure of these polysaccharides in greater detail Liver and muscle glycogen from rabbits and corn and wheat amylopectin were exhaustively treated first with crystalline muscle phosphorylase in a medium of inorganic phosphate This treatment degraded the branches outside of the first tier of branch points, forming α-D-glucose 1-phosphate and leaving a limit dextrin, LD_1. It should be noted that the two outer branches arising from each branch point are

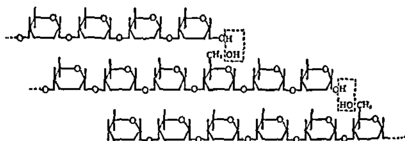


FIG 10 Haworth and Hirst's "laminated" formula for starch or glycogen

unequally attacked by phosphorylase. One branch is completely degraded, leaving the one glucose unit in 1,6-linkage exposed, whereas the other branch is degraded down only to 5 or 6 D-glucose units (Fig. 11) The glucosidase appears to be highly specific for this structure and splits off only the 1,6-linked residue as D-glucose

The limit dextrins, LD's, so formed were isolated and submitted to the action of amylo-1,6-glucosidase in a phosphate-free medium until the reaction had come to an end After inactivation of glucosidase by heating, the products were treated again with phosphorylase and the second limit dextrins, LD_2, were isolated. Several such separate degradations were repeated until 70% to 90% of the original polysaccharides had been digested Portions of the limit dextrins were also analyzed for per cent end groups and for the degradation limit with β-amylase. The results indicated that the branched polysaccharides differ in the lengths of the outer and inner chains and in the number of tiers of branch points Glycogen has outer chains that average 7 to 13 D-glucose units, inner chains averaging 3 to 6, and a total of 5 to 7 tiers Starch amylopectin appears to be very similar, with outer chains of 13 to 16 D-glucose units, inner chains of 5 to 6, and 4 to 5 tiers

In consideration of results of β-amylolytic degradation and unit-chain degree of polymerization for a number of different amylopectins, Manners (*164*) concluded that the outer chains vary in length from 13 to 14 to

18 to 19 D-glucose units according to the source of the amylopectin, the inner chains varying in length from 3 to 4 to 8 to 9 D-glucose units.

Glycogen is branched to a larger degree than amylopectin, which means that the proportion of the 1,6-α- to 1,4-α-linkages is greater in the former polysaccharide than in the latter "The degree of branching" (relative proportion of 1,6- to 1,4-linkages) is determined from the percentage of end groups which can now be estimated by two independent

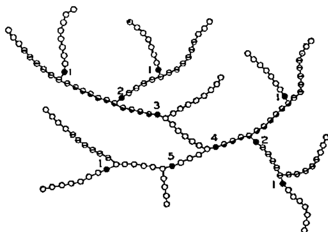


FIG 11 Fragment of muscle glycogen based on the results obtained by stepwise enzymic degradation

○, ⊖, ⊖ glucose residues removed by first, second, and third digestions with phosphorylase, respectively ●, glucose residue split off as free glucose from 1,6-linkage by amylo-1,6-glucosidase.

methods (1) Haworth's methylation technique, and (2) periodate oxidation In the two chemical methods, the nonreducing terminal D-glucose units of the outer branches yield tetramethylglucose in the methylation procedure, whereas formic acid is obtained in the periodate oxidation method The number of end groups is related to the number of branch points as n is related to n-1 In the enzymic method introduced by Cori (*143*) in which amylo-1,6-glucosidase is used, it is not the terminal nonreducing D-glucose but the D-glucose residues in 1,6-α-linkage that are determined as free D-glucose.

For example, when amylopectin is assayed for end group by either of the two chemical methods, or for branch points by the enzymic method, a value of about 5% is obtained. This means that, on the average, there is one 1,6-linkage per every nineteen 1,4-linkages in the molecule. The

total value of twenty, termed "average chain length," comprises the average length in D-glucose units of the outer and inner branches. The "degree of branching" is thus inversely proportional to the "average chain length."

The analytical data obtained after each successive enzymic degradation of glycogen or amylopectin (*153*) could fit only a model representing a multibranched polysaccharide similar to that proposed by Meyer (*149, 150*). If the structure were "laminated" like the one formulated by Haworth *et al.* (*147, 148*), successive enzymic degradation would yield a constant percentage of the total branch points in each tier. Actually a diminishing percentage of the total branch points is obtained as the polysaccharide is degraded from tier to tier.

Taking the molecular weight of glycogen to be about one million, with an average chain length of 12 D-glucose units, the molecule should contain approximately 500 branch points. In subjecting a "laminated" molecule to the action of muscle phosphorylase, only the first two branches (Fig. 10, top) having a common branch point, would result in an exposed 1,6-glucose unit and a stub consisting of 5 or 6 D-glucose units. All the rest of the branches would be degraded by this enzyme, leaving similar stubs, but no exposed single D-glucose units. After the first degradation with phosphorylase, the resulting product would be a 1,4-linked linear chain with 1,6-intermittent linkages approximately every sixth D-glucose unit, with short stubs attached to each of the linkages. There would thus be only one 1,6-linked D-glucose unit exposed and subject to the attack of the amylo-1,6-glucosidase. Repetition of this treatment with phosphorylase would leave one such unit each time. A "laminated" structure would thus require several hundred consecutive enzymic treatments to degrade the polysaccharide to 70% or 90%. Actually four or five degradations are sufficient to break down the glycogen to that extent; this is in agreement with a multibranched or tree-like structure. If this structure were completely regular, 50% of all the branch points would be in the first tier, 25% in the second, 12.5% in the third, etc. In experiments with a number of glycogens from various sources, successive treatment with 1,6-glucosidase produced amounts of free D-glucose that approximated these values. Similar results were obtained when amylopectin was subjected to the same analysis. It can be concluded from these considerations that a "laminated" model for glycogen or amylopectin is untenable.

Additional evidence in support of the multibranched structure of amylopectin was presented by Peat *et al.* (*155*). They submitted waxy maize starch (amylopectin) to β-amylase degradation and then hydrolyzed the residual dextrin with R-enzyme, which degrades 1,6-linkages

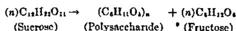
Consideration of the structure, based on a quantitative evaluation of the products obtained, also led them to decide in favor of a multibranched (Fig 6) rather than a laminated pattern

VII. Synthesis of Starch-Glycogen Type Polysaccharides by Transglycosylases

A Amylopectin-Type Polysaccharide from Sucrose

Hehre and co-workers (*156–158*) found that cultures, washed cells, and enzyme preparations of *Neisseria perflava*, isolated from the human throat or nasopharynx, produce amylopectin- or glycogen-like polysaccharide from sucrose The polysaccharide gives a maroon color with iodine, and is degraded with β-amylase and crystalline muscle phosphorylase to about the same extent as amylopectin, it forms an insoluble butanol complex with iodine, and gives a negative serological test for dextran Data obtained from methylation experiments (*159*) show that the polysaccharide consists of chains averaging 11 to 12 1,4-α-glucopyranose units in length, and that the branches are combined by linkages of the 1,6-type The enzyme system responsible for the synthesis of this polysaccharide from sucrose is known as "amylosucrase" (*160*)

Inasmuch as the polysaccharide is branched, it must be assumed that another enzyme is present, which in collaboration with the amylosucrase synthesizes a branched structure This assumption is confirmed by the observation that the *N perflava* preparations have the ability to convert amylose to an amylopectin-type polysaccharide without the production of reducing sugars The cell-free enzyme preparation catalyzes the reaction involving the substitution of chiefly 1,4-linkages in the polysaccharide chain for the 1,2-linkage in sucrose



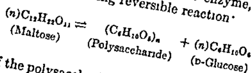
$$\underset{\text{(Sucrose)}}{(n)C_{12}H_{22}O_{11}} \rightarrow \underset{\text{(Polysaccharide)}}{(C_6H_{10}O_5)_n} + \underset{\text{(Fructose)}}{(n)C_6H_{12}O_6}$$

When cultures or enzyme preparations of *N. perflava* are allowed to act on α-D-glucose 1-phosphate, some amylopolysaccharide is produced, indicating the presence of a phosphorylase However, the amylosucrase can be distinguished from the bacterial phosphorylase by its stability to heat and by the fact that the synthesis of the polysaccharide from sucrose is not suppressed in high concentration of inorganic phosphate. The synthetic reaction proceeds without a noticeable lag period, suggesting that primer material, if needed, is present in the preparation Although there is no direct evidence for this, the fact that the formation of polysaccharide is completely inhibited in the presence of traces of salivary amylase indicates that a primer may be required.

Inasmuch as the molar concentration of polysaccharide is extremely low because of its high molecular weight, there is a strong tendency for the reaction to go to the right For this reason it is difficult to demonstrate the reverse reaction However, Hehre and Hamilton (*161*) were able to show that a small amount of polysaccharide, possessing the serological properties of dextran, is formed when a mixture of starch and D-fructose is subjected to the action of amylosucrase and dextransucrase, which has the ability to convert sucrose to dextran

B SYNTHESIS OF AMYLOSE FROM MALTOSE

Monod and Torriani (*162–164*) obtained a cell-free enzyme preparation from a special variant strain of *Escherichia coli* that converts maltose into a starch-like polysaccharide and D-glucose The enzyme shows a high degree of specificity, inasmuch as neither sucrose, α-D-glucose 1-phosphate, lactose, melibiose, cellobiose, nor methyl α- or β-D-glucoside can be used as substrate They showed that this enzyme, named "amylomaltase," catalyzes the following reversible reaction·



$$(n)C_{12}H_{22}O_{11} \rightleftharpoons (C_6H_{10}O_5)_n + (n)C_6H_{10}O_6$$

(Maltose) (Polysaccharide) (D-Glucose)

The nature of the polysaccharide formed by the amylomaltase depends upon the concentration of D-glucose in the reaction Equilibrium is established when approximately 60% of the maltose has been degraded. The product produced in the reaction stains faintly red with iodine, indicating that the polymeric material consists of short-chain dextrins However, if the D-glucose is continually removed with D-glucose oxidase, equilibrium can never be established, and the conversion of maltose proceeds to completion. Under these conditions, the product stains deep blue with iodine, indicating that it is at least partially amylose. The effect of the presence of D-glucose on the molecular size of the polysaccharide can be interpreted in terms of the reversibility of the above reaction By removing the D-glucose the equilibrium would be expected to shift to the right, whereas in the presence of D-glucose, the reverse reaction would lead to the partial depolymerization of the polysaccharide

Doudoroff *et al* (*165*) independently found that another variant of *E coli* catalyzes the same type of nonphosphorolytic reaction When D-glucose is allowed to accumulate during the decomposition of maltose, the polysaccharide produced by this enzyme consists of reducing dextrins composed on the average of from 4 to 6 D-glucose units Working with Monod's strain of *E. coli*, Barker and Bourne (*166*) fractionated the products produced from maltose on a charcoal column. They showed

that these products consisted of D-glucose, unchanged maltose, and higher homologs of maltose composed of from 3 to 5 D-glucose units in length

Since equivalent amounts of polysaccharide and D-glucose are formed from maltose *in vitro* without the intervention of inorganic phosphate, a phosphorolytic mechanism for the amylomaltase reaction is excluded The mechanism appears to be a direct transfer of D-glucose units from one molecule to another. Although evidence is still lacking, it seems probable that the first step in the formation of the polysaccharide chain is the transfer of one nonreducing moiety of a maltose molecule to another, forming maltotriose The chain is then lengthened by repetition of the transglucosylation reaction

Besides the enzyme, amylomaltase, the *E coli* also contains phosphorylase, phosphoglucomutase, and phosphohexoisomerase (*165*) Owing to the reversible nature of the amylomaltase reaction, maltose and reducing dextrins are produced when α-D-glucose 1-phosphate and D-glucose are added to the preparations In the absence of glucose only a starch-like polysaccharide is formed from α-D-glucose 1-phosphate

$$\text{Maltose} \underset{}{\overset{\text{amylomaltase}}{\rightleftharpoons}} \text{polysaccharide} + \text{D-glucose}$$
$$\updownarrow \text{phosphorylase}$$
$$\alpha\text{-D-glucose 1-phosphate}$$

It appears that an identical polysaccharide is formed in the same organism by two different enzymic mechanisms, namely one involving a transglucosylase and the other a phosphorylase

C Formation and Degradation of Cyclic Amylosaccharides

Schardinger (*167*) observed in 1905 that when starch solutions are exposed to the action of the microorganism *Bacillus macerans*, a mixture of water-soluble dextrins is produced From this mixture three distinct, nonreducing crystalline compounds (Schardinger dextrins) can be isolated These dextrins, α, β, and γ, are now known to possess cyclic structures consisting of six, seven, and eight 1,4-α-glucosidically linked D-glucose units, respectively (*168–170*) Hudson *et al* (*171, 172*) showed that the dextrins are produced by an extracellular enzyme of the *B. macerans* microorganism, and probably arise from the linear amylose starch fraction and from the outer branches of amylopectin Schwimmer and Garibaldi (*173*) succeeded in separating the enzyme in electrophoretically pure form

In forming cyclic dextrins from starch, the *B macerans* enzyme is apparently capable of bringing about an exchange of energy from one 1,4-α-glucosidic bond in a linear chain to another new bond of the same chain in a cyclic dextrin The enzyme, therefore, belongs in the group

appears to synthesize cellulose in the absence of UDP-D-glucose. The role of sugar nucleotide in the synthesis of cellulose is still not clear.

Feingold *et al* (*38*) have shown that a solubilized enzyme preparation obtained from mung bean particles will catalyze the formation of a water-soluble radioactive polysaccharide from C^{14}-labeled UDP-D-glucose with the liberation of uridine diphosphate. The reaction for polymer formation required an activator, which did not appear to be incorporated into the product D-Glucose and a number of D-glucosides were able to serve as activators

Partial hydrolysis of the polymer with acid resulted in the formation of a series of oligosaccharides of degree of polymerization from 2 to 8, which were chromatographically identical with those present in a partial hydrolyzate of the β-1,3-linked polysaccharide, laminaran Chemical degradation of the disaccharide with lead tetraacetate produced arabinose (*181*), conclusively proving that the disaccharide is linked between the C-1 and C-3 positions of the two D-glucose units The oligosaccharides obtained from the synthetic polymer were completely hydrolyzed with emulsin to D-glucose, indicating that the glycosidic linkages are of the β-type The D-glucose residues in the polysaccharide are therefore joined by β-1,3-glycosidic linkages Here again the reaction of polysaccharide formation proceeds with an inversion of the α- to a β-configuration.

β-1,3-linked D-glucose polysaccharides are known to be constituents of *Laminaria* (laminaran) and of yeast cell walls (glucan) (*182*). Recently, Aspinall and Kessler (*183*) showed that the polysaccharide "callose" isolated from the sieve plates of phloem of grape vines is a β-1,3-linked glucan While it was previously thought that callose was predominantly localized in the phloem tissue and in pollen (*184*), it now appears that this polysaccharide is readily produced in other higher plant tissues as a result of injury to the cells (*185*)

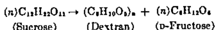
IX. Synthesis of Dextran

A Dextran from Sucrose

Growing cultures of *Leuconostoc mesenteroides, Leuconostoc dextranicum*, and *Betabacterium vermiforme* are capable of synthesizing dextrans from sucrose (*160–190*). These dextrans are polymers of D-glucose residues which are combined chiefly by 1,6-α-linkages. Hehre (*160*) and his collaborators showed that dextran can also be synthesized from sucrose by cell-free extracts of *Leuconostoc*. The enzyme of these microorganisms which is responsible for the synthesis of dextran was named "dextransucrase" During World War II dextran assumed considerable importance, inasmuch as it was extensively used as a blood plasma substitute.

Sucrose is a highly specific substrate for the enzyme, which is produced only by bacteria grown on this disaccharide When a crude dextransucrase preparation is inoculated with sucrose, the synthesis of dextran is indicated by serological testing (*191*), development of opalescence, formation of an alcohol-precipitable polysaccharide, and accumulation of D-fructose The dextrans produced by different species of bacteria vary considerably in their molecular constitution Thus, the one synthesized by *Leuconostoc dextranicum* appears to be essentially unbranched, whereas those obtained by the action of other microorganisms are highly branched (*186–190*) The branches are generally connected by 1,4-linkages, but in some cases linkages of the 1,3-type are found (*193–194*) In the polysaccharides where branching occurs, it is necessary to assume that in addition to dextransucrase, which forms the main 1,6-linkage, another enzyme responsible for the establishment of the 1,4- or 1,3-linkage is present.

Leuconostoc mesenteroides also contains a sucrose phosphorylase However, since it has been demonstrated that sucrose is converted to dextran by dextransucrase in the absence of a detectable quantity of inorganic phosphate and that the enzyme does not form polysaccharide from α-D-glucose 1-phosphate, it is clear that phosphorylase is not involved in dextran synthesis The evidence indicates that the dextransucrase acts by a direct transfer of D-glucose units The reaction appears to involve the substitution of a 1,6-glucosidic linkage for a glucose-fructose bond and can be represented by the following equation:



$$\underset{\text{(Sucrose)}}{(n)C_{12}H_{22}O_{11}} \rightarrow \underset{\text{(Dextran)}}{(C_6H_{10}O_5)_n} + \underset{\text{(D-Fructose)}}{(n)C_6H_{12}O_6}$$

When dextransucrase is allowed to act on sucrose, the final concentration of the disaccharide becomes extremely low Attempts to demonstrate the reversibility of the reaction by showing a formation of sucrose in the reaction medium when the enzyme is incubated with D-fructose and dextran were not successful

Stodola *et al* (*195*) found a new reducing disaccharide, 5-*O*-D-glucopyranosyl D-fructopyranose, named leucrose, which is formed in the reaction mixture to the extent of about 3% during the synthesis of dextran from sucrose Koepsell and co-workers (*196*) further demonstrated that when certain sugars, notably isomaltose, maltose, methyl-α-D-glucoside, and D-glucose, are added to dextran-synthesizing reaction mixtures, oligosaccharides are produced as the major products, instead of high molecular weight dextran These sugars appear to act as glycosyl acceptors, alternate with the normal acceptor (the nature of the normal acceptor is not known at present), and initiate chain formation. They seem to perform a function in dextran synthesis analogous to that of

primer in starch synthesis. The oligosaccharides produced comprise the series expected to be formed from successive addition of D-glucose by 1,6-α-glucosidic linkage to the alternate acceptor. D-Fructose, leucrose, melibiose, and D-galactose also act as glucosyl acceptors but are much less efficient

B Dextran from Amylodextrin

Hehre and Hamilton (*197*) demonstrated that certain acetic acid bacteria, *Acetobacter viscosum* and *A capsulatum*, are also capable of forming dextran. Unlike *Leuconostoc mesenteroides*, these bacteria do not produce polysaccharide from sucrose but require dextrin as a spec substrate The enzyme responsible for this reaction (dextrandextrina has been obtained free from bacterial cells The conversion of dext to dextran presumably involves the conversion of 1,4-glucosidic bon to 1,6-bonds

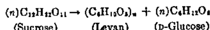
Dextran formation occurs only from certain dextrins No synthesis has been observed from amylose, amylopectin, glycogen, β-limit dextrins, Schardinger dextrin, maltose, sucrose, or α-D-glucose 1-phosphate The fact that dextran is formed from amyloheptaose (*169*) or from products of acid or α-amylase hydrolyzates of amylose (*198*) indicates that the essential action of the dextrandextrinase is the conversion of 1,4-glucosidic bonds to 1,6-bonds When acting on dextrins or amyloheptaose the substrate disappears to the extent that dextran is formed. The enzyme appears to catalyze the repetitive transfer of α-D-glucopyranosyl units from the nonreducing end of a dextrin, establishing branch points in a growing dextran molecule

X. Synthesis of Levan from Sucrose

In 1910 Beijerinck (*199*), working with *Bacillus megaterium* and certain other bacteria, was first to observe polysaccharide formation by the agency of an extracellular enzyme When the microorganisms were grown on agar plates containing sucrose, microscopically visible particles of levan (a polyfructofuranose in which the main glycosidic linkages are of the 2,6-type) appeared in the agar. He declared the active principle to be a synthetically active enzyme which he termed "viscosaccharase" He further established the enzymic nature of the reaction by showing that the active agent responsible for the synthesis of the polysaccharide is diffusible on a sterile agar plate and that it can be inactivated by heat

Hestrin and his co-workers (*200, 201*) succeeded in liberating the enzyme (levansucrase) from washed cells of *Acetobacter levanicum* by autolysis in the presence of thymol and chloroform, and showed that it

converts sucrose to levan and D-glucose In this reaction a 2,6-linkage is substituted for the glycosidic bond in sucrose, forming the levan:



$$(n)C_{12}H_{22}O_{11} \rightarrow (C_6H_{10}O_5)_n + (n)C_6H_{12}O_6$$
(Sucrose) (Levan) (D-Glucose)

Similarly, raffinose is converted by this enzyme to levan and melibiose.

Levan synthesis from sucrose was found to be strongly inhibited by D-glucose, D-galactose, D-xylose, L-arabinose, maltose, and lactose, but not by D-mannose, D-fructose, or D-glucosamine The inhibitory action of these sugars was attributed chiefly to the configuration of carbon atom 2 of the reducing carbon chain of the molecule

Inorganic phosphate is not required for levan synthesis, and it has no effect on the rate of reaction, no esterification of phosphate can be detected It is therefore considered that levan synthesis is the result of the transfer of D-fructose units from sucrose to a suitable acceptor through the mediation of a fructose-enzyme complex

The molecular weights of levans are extremely high Feingold and Gehatia (*202*) determined the molecular weight of native levan of *Acetobacter levanicum* cultures to be 17 million, and of enzymically synthesized levan 67 million Dedonder and Slizewicz (*203*) report molecular weight values for levans produced by *Bacillus subtilis* and for levans which were subjected to mild acid hydrolysis, from a thousand to over 100 million

Murphy (*204*), in his investigation of *Bacillus polymyxa* levan by the methylation method, detected 3,4-*O*-dimethyl-D-fructose in the products of hydrolysis of the methylated polysaccharide, indicating that the levan is branched Bell and Dedonder (*205*) found that the levans produced by *Pseudomonas prunicola* and *Bacillus subtilis* contained branches of an average chain length of 9-10 D-fructofuranose residues combined through β-2,6-linkages They found that these levans are highly branched Feingold and Gehatia (*202*) showed that levan synthesized by cell-free preparations of *Acetobacter levanicum*, which had a molecular weight of 67 million, was branched, the branch linkages being through C-2 and C-1, with branches of an average chain length of 9 fructosyl units, and main linkages between C-2 and C-6

Because of the extremely high molecular weight of levan, its concentration in solution is very low, which is probably the reason why the reverse reaction of levan formation, i e , the production of sucrose from native levan and D-glucose, cannot be demonstrated However, Péaud-Lenoël and Dedonder (*206*) showed that when levan is degraded to a molecular weight of approximately 5,500, the synthesis of this polysac-

charide from sucrose in the presence of levansucrase is reversible The equilibrium constant of this reaction at 37° and pH 6 2 was found to be $K = 0.037$. From this equilibrium constant the energy of the fructosyl bond in the polysaccharide was calculated to be approximately 4000

Péaud-Lenoel (*207*) found that levan acts as a precursor of its own synthesis from sucrose in the presence of levansucrase, and that the Michaelis and Menten constant for the enzyme-sucrose complex at 37° and pH 6 2 is 9×10^{-2} *M* This author also showed that levan behaves as an activator of its own synthesis, acting as noncompetitive inhibitor of the formation of free D-fructose by the same enzyme. He demonstrated the existence of a specific enzyme-levan complex, $K_L = 10 \times 10^{-3}$ *M*

Hestrin *et al* (*208*), on the basis of a study of the action of levansucrase on a number of oligosaccharides, concluded that those compounds containing a terminal β-D-fructofuranosyl group linked to an aldose, as in sucrose (α-D-glucopyranosyl β-D-fructofuranoside), sucrose analogs differing from sucrose in the aldosyl moiety, and raffinose, are capable of forming levan An exception to this rule was found in the case of umbelliferose [(α-D-galactopyranosyl)-(1 → 2)-*O*-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside], in which the carbon adjacent to the anomeric carbon of the glucosyl moiety of sucrose is substituted by D-galactose (*209*) *Umbelliferose is also not attacked by yeast invertase.* Oligosaccharides with a terminal β-D-fructofuranosyl attached to a carbinol carbon, such as 6-*O*-β-D-fructofuranosyl D-glucose, are not attacked by this enzyme

Substitution of the β-D-fructofuranosyl moiety in sucrose, at position C-1, C-3, or C-6 by a glycosyl group leads to a loss of capacity to form levan by levansucrase The trisaccharide, melezitose, which contains sucrose and in which C-3 of the β-D-fructofuranosyl moiety is substituted by D-glucose, may be cited as an example

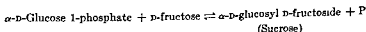
XI. Mechanisms of Formation of β-Linked Saccharides

It has been established that in the phosphorolysis reactions, with muscle or potato phosphorylase, where the phosphate bonds of the α-D-glucose 1-phosphate are exchanged for glucosidic bonds, forming a polysaccharide, the α-type linkage is preserved in the latter compound.



α-D-Glucose 1-phosphate ⇌ α-polysaccharide + P

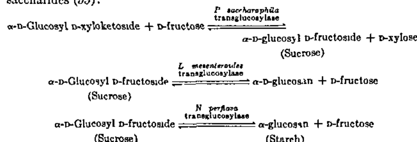
The same can be observed in the phosphorolysis of sucrose with sucrose phosphorylase.



α-D-Glucose 1-phosphate + D-fructose ⇌ α-D-glucosyl D-fructoside + P
(Sucrose)

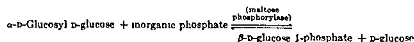
In the transglycosylation reactions in which sucrose (*210*), sucrose phosphate (*211*), and trehalose phosphate (*212*) are formed from UDP-D-glucose as the common D-glucose donor, and D-fructose, D-fructose 6-phosphate, and D-glucose 6-phosphate as the second monosaccharide moiety, respectively, the α-linkage is also unchanged

Similarly, in the transglycosylation reactions in which disaccharides or polysaccharides are formed through exchange of glycosidic linkages in a number of disaccharides and polysaccharides, no change of linkage occurs The following are examples in which transglycosylases from the microorganisms *P saccharophila*, *L mesenteroides*, and *N. perflava* were used on substrates containing α-glucosidic linkages to form complex saccharides (*53*).



Here, again, we find the same α-type linkage in the products as in the substrates

Fitting and Doudoroff (*213*) were first to observe a new type of enzymic reaction in which the enzyme causes an inversion of the type of glycosidic linkage when synthesis or degradation takes place They found that the bacterium *Neisseria meningitidis* contains an enzyme, maltose phosphorylase, capable of catalyzing the reversible reaction·

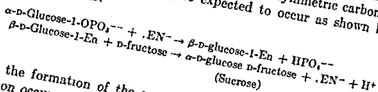


Starting with maltose and inorganic phosphate, the enzyme produces β-D-glucose 1-phosphate + D-glucose In the reverse reaction, β-D-glucose 1-phosphate and D-glucose form inorganic phosphate and maltose, in which the α-type linkage is preserved. The reaction does not take place when the α-ester is substituted for the β-ester. D-Xylose was the only monosaccharide that reacted with the β-D-glucose 1-phosphate in the presence of maltose phosphorylase, producing a reducing disaccharide which consisted of D-glucose and D-xylose.

More recently several other transglycosylation reactions have been discovered in which β-linked complex saccharide is formed from an α-linked substrate, i.e., chitin (*177*) from UDP-*N*-acetyl-D-glucosamine, callose (*38*) from UDP-D-glucose, and glycosides (*214*) from UDP-glucuronic acid.

It is of interest to consider the differences that distinguish the reaction of maltose formation by *Neisseria meningitidis*, in which a change of the type of linkage occurs, from other phosphorolytic or transglycosylitic reactions in which the type of glycosidic linkage is preserved during the reaction.

Koshland (*215*) recently proposed a model for the reaction involving the formation of intermediate enzyme-substrates, in particular for the acetyl-CoA reactions and for the sucrose phosphorylase reaction, which has a glucose-enzyme intermediate and undergoes cleavage of the C—O bond. He postulates that the enzyme or coenzyme contains a group, $.En^{-}$, having a reactive electron pair. This electron-sharing group is capable of attacking a saturated asymmetric carbon atom in α-D-glucose 1-phosphate. In nonenzymic reactions this type of displacement is known to cause an inversion in the configuration of the asymmetric carbon atom. The reaction might, therefore, be expected to occur as shown by the following equations:



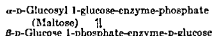
$$\alpha\text{-D-Glucose-1-}OPO_3^{--} + .EN^{-} \rightarrow \beta\text{-D-glucose-1-En} + HPO_4^{--}$$

$$\beta\text{-D-Glucose-1-En} + \text{D-fructose} \rightarrow \underset{\text{(Sucrose)}}{\alpha\text{-D-glucose D-fructose}} + .EN^{-} + H^{+}$$

In the formation of the sucrose phosphorylase-glucose complex, inversion occurs to produce the β-form of α-D-glucose 1-phosphate. The D-fructose molecule then displaces the enzyme from the intermediate, again with inversion, to give the sucrose molecule having the α-configuration. The phosphorolysis of sucrose thus represents a double Walden inversion. According to Koshland this model suggests an explanation for the retention of the configuration, the formation of enzyme intermediate, and the carbon-oxygen cleavage by mechanisms which are well established in nonenzymic reactions.

We know that the sucrose-synthesizing enzyme acts as D-glucose donor and acceptor to its substrate, functioning both as a phosphorylase and transglucosylase. The enzyme reacts with the D-glucose moiety of α-D-glucose 1-phosphate, forming a glucose-enzyme intermediate and then donates it to D-fructose, producing sucrose. The sucrose phosphorylase is also capable of accepting the D-glucose moiety of a disaccharide such as α-D-glucosylsorboside and transferring the D-glucose unit of this disaccharide to D-fructose, forming sucrose.

Fitting and Doudoroff (*213*) found that in the maltose phosphorylase reaction no exchange between phosphate or arsenate and β-D-glucose 1-phosphate occurs. Neither does an exchange take place between maltose and D-glucose. This seems to distinguish the mechanisms of catalysis of the phosphorolyses of maltose and sucrose. In the case of maltose phosphorylase the enzyme itself does not appear to be a carrier of the glucosidic moiety. Both donor and acceptor appear to be necessary components for the catalysis of the D-glucose transfer to take place. A scheme which accounts for the necessity of the three components would involve the formation of a maltose-enzyme-phosphate complex interconvertible with a β-D-glucose 1-phosphate-enzyme-glucose complex as an intermediate:



The observed single inversion in the phosphorolysis of maltose may be explained on the basis of a three-component system in which the phosphate transfer occurs between the substrates rather than between the enzyme and each substrate separately. The occurrence of the inversion would indicate that the carbon-oxygen bond of the phosphate ester is broken, as has been shown in the case of sucrose phosphorylase (*56*).

Such a mechanism of enzyme action may explain the fact that when starch is hydrolyzed with β-amylase, upward mutarotation is observed, indicating that the amylase liberates maltose in the β-configuration.

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CHAPTER 7

Fatty Acid Oxidation and Synthesis

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I. Fatty Acid Oxidation

A Introduction

The quest for an understanding of the mechanism of fatty acid oxidation which began with the studies of Knoop (*1*) in 1904 on hippuric acid formation from phenyl fatty acids, has extended over a span of half a century. A step-by-step advance had to be made from more to less complex experimental systems—from whole animal studies to perfused organs, from tissue slices to homogenates, and finally from mitochondria to soluble enzymes These transitional stages in the development of our knowledge which led finally to the elucidation of mechanism in terms of intermediates, coenzymes, and reaction sequences, at the same time also mirror the various phases in the maturing of biochemical thinking and methodology.

Knoop (*1*) first proposed the idea that fatty acids are degraded by

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Knoop (*1*) first proposed the idea that fatty acids are degraded by

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repetition of a β-oxidation cycle in which acetate and a new fatty acid with two carbon atoms less than the parent fatty acid are formed It may at first glance be difficult to understand why fifty years had to elapse before this hypothesis could be verified by experiment The explanation lies in the fact that fatty acid oxidation *in vivo* proceeds in such fashion that no intermediates between the fatty acid and the final products of oxidation (CO_2 and water) accumulate. Acetoacetate, strictly speaking, is not an intermediate but an end product of a partial or incomplete oxidation of fatty acids This crippling difficulty in the way of penetrating into the mechanism of oxidation applied at all levels from the whole animal to the cell-free mitochondrion. Only at the level of the isolated soluble enzyme did it become possible to interrupt the sequence in such a way as to accumulate intermediates. In studies of multienzyme sequences this difficulty is not unique. Whenever the intermediates are linked to coenzymes or enzymes present in catalytic amounts, then accumulation of more than trace amounts of intermediates is of course interdicted

Fatty acids (or their derivatives) are oxidized only in the form of their corresponding esters with coenzyme A formed by the interaction of the carboxyl group of the fatty acid with the SH-group of coenzyme A. Not until coenzyme A had been discovered in the laboratory of F. Lipmann (*2*) and then later made available as a reagent which could be added in substrate amounts (*3*), was it possible to approach the problem of intermediates In 1951, some five years after the discovery of coenzyme A, Lynen and Reichert (*4*) of Munich reported the isolation of acetyl coenzyme A from yeast and the characterization of active acetate as a thiol ester of acetic acid and coenzyme A In 1953 as a consequence of the discovery of a unique and specific method for precipitating coenzyme A from crude tissue or cell extracts, highly purified coenzyme A could for the first time be isolated in gram quantities (*3*). No major obstacle then remained in the way of reconstructing fatty acid oxidation in a system of soluble enzymes. Within the same year this objective was attained simultaneously by two groups—by Lynen of the Munich laboratory collaborating with Ochoa of New York University (*5*) and by Green (*6*) and his colleagues in the Madison laboratory.

Mitochondria of animal tissues contain the full complement of enzymes which catalyze the oxidation of fatty acids to carbon dioxide and water ultimately by way of the citric acid cycle (*7*, *8*). The use of mitochondrial suspensions as starting material simplified enormously both the search for and the preparation of the component enzymes which intervene in the over-all process of fatty acid oxidation When an acetone powder of mitochondria is extracted with dilute salt solutions, the

clarified extract is found to contain all the enzymes of the fa oxidation system in soluble and relatively concentrated form (9,

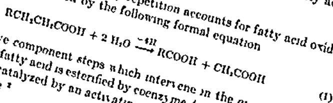
While the studies carried out with isolated enzyme system defined the number and nature of the enzymes, coenzymes, and mediates concerned in fatty acid oxidation, they have by no clarified some important aspects of the physiological process as it ceeds in the intact mitochondrion Studies of soluble systems thro light on questions such as the intramitochondrial arrangement of enzymes of the fatty acid oxidation cycle (a) with respect to one anoth and (b) with respect to bound cofactors such as DPN, ATP, and Co Such information can only be garnered from direct studies of the mito chondrion or at least on particulate submitochondrial fragments that sti contain the full complement of enzymes for the oxidation of fatty acids.

B The Fatty Acid Oxidation Cycle

The basic sequence which by repetition accounts for fatty acid oxidation may be represented by the following formal equation

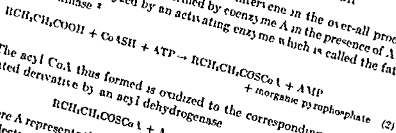
$$RCH_2CH_2COOH + 2\,H_2O \xrightarrow{-4H} RCOOH + CH_3COOH \qquad (1)$$

There are five component steps which intervene in the over-all process First the free fatty acid is esterified by coenzyme A in the presence of ATP —a reaction catalyzed by an activating enzyme which is called the fatty acid thiokinase [2]



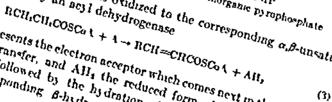
$$RCH_2CH_2COOH + CoASH + ATP \rightarrow RCH_2CH_2COSCoA + AMP + \text{inorganic pyrophosphate} \qquad (2)$$

The acyl CoA thus formed is oxidized to the corresponding α,β-unsaturated derivative by an acyl dehydrogenase

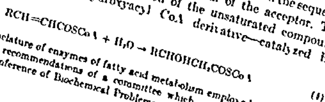


$$RCH_2CH_2COSCoA + A \rightarrow RCH{=}CHCOSCoA + AH_2 \qquad (3)$$

where A represents the electron acceptor which comes next in the sequence of electron transfer, and AH_2 the reduced form of the acceptor. The oxidation is followed by the hydration of the unsaturated compound to the corresponding β-hydroxyacyl CoA derivative—catalyzed by enoyl hydrase



$$RCH{=}CHCOSCoA + H_2O \rightarrow RCHOHCH_2COSCoA \qquad (4)$$



[2] The nomenclature of enzymes of fatty acid metabolism employed in this review conform to the recommendations of a committee which convened at the second International Conference of Biochemical Problems of Lipids Ghent, Belgium, July, 1955 (11)

A second dehydrogenation then takes place—*this time at the β-carbon* atom—which is catalyzed by β-hydroxyacyl dehydrogenase with DPN as specific electron acceptor:



$$RCHOHCH_2COSCoA + DPN^+ \rightarrow RCOCH_2COSCoA + DPNH + H^+ \quad (5)$$

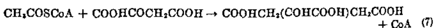
Finally, β-ketoacyl thiolase cleaves the β-ketoacyl derivative according to the following equation:



$$RCOCH_2COSCoA + CoASH \rightarrow RCOSCoA + CH_3COSCoA \quad (6)$$

The sum of Eqs (2–6) is in fact equivalent to (1) except that RCOOH and the C_2 unit in the latter equation are represented as the free fatty acids rather than as their esters of coenzyme A At the completion of each sequence represented by Eqs (2–6) the fatty acid is degraded by two carbon atoms A new fatty acid is thus generated which can then in turn undergo a repeat of the degradation process

Acetyl CoA, the C_2 unit which is the end product of each degradative sequence condenses with oxalacetate to form citrate in presence of a specific condensing enzyme which has been thoroughly studied by the Ochoa school (*12*).



$$CH_3COSCoA + COOHCOCH_2COOH \rightarrow COOHCH_2(COHCOOH)CH_2COOH + CoA \quad (7)$$

Since citrate is oxidized eventually to CO_2 and water by way of the citric acid cycle, the reaction described by Eq (7) may be looked upon *as the one linking the two key mitochondrial cycles*—the fatty acid and the citric acid cycles

The six basic enzymic reactions which make up the fatty acid oxidation sequence are thus. (1) acylation of CoA by the fatty acid· (2) α,β-dehydrogenation, (3) hydration of the α,β-unsaturated derivative, (4) β-oxidation of the β-hydroxyacyl CoA to the corresponding β-ketoacyl CoA; (5) thiolytic cleavage of the β-ketoacyl CoA to a fatty acyl CoA and acetyl CoA; and finally (6) condensation of acetyl CoA and oxalacetate to form citrate.

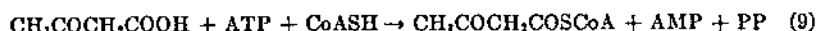
There are a few additional enzymic reactions which have to be considered in the context of fatty acid oxidation In liver mitochondria fatty acid oxidation proceeds partly to completion, i e, to CO_2 and water, while the rest goes no further than to the stage of acetoacetate. The accumulation of acetoacetate as an end product of fatty acid oxidation in liver is the consequence of the presence of an enzyme system which in

effect catalyzes the deacylation of *S*-acetoacetyl CoA to acetoacetate and CoASH[3] (*17–19*).



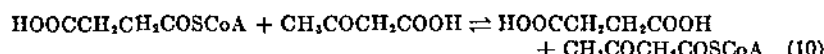
$$CH_3COCH_2COSCoA + H_2O \rightarrow CH_3COCH_2COOH + CoASH \quad (8)$$

Since the deacylation reaction is irreversible and furthermore, since liver is deficient or lacking in the enzyme which catalyzes the acylation of CoA by acetoacetate (*10*),



$$CH_3COCH_2COOH + ATP + CoASH \rightarrow CH_3COCH_2COSCoA + AMP + PP \quad (9)$$

acetoacetate accumulates in liver and not in other tissues It is formed in other tissues, but does not accumulate because of one or both of two active processes: (1) the ATP-catalyzed conversion to the acyl CoA as in Eq (9), (2) the succinyl CoA-catalyzed conversion of acetoacetate to the acyl CoA by thiophorase—an enzyme which labilizes the thioester bond of acyl CoA esters and catalyzes the replacement of succinyl by acetoacetate or acetoacetyl by succinate (*20*, *21*), Eq (10)·

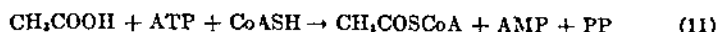


$$HOOCCH_2CH_2COSCoA + CH_3COCH_2COOH \rightleftharpoons HOOCCH_2CH_2COOH + CH_3COCH_2COSCoA \quad (10)$$

C Preparation of Acyl Coenzyme A Derivatives

The availability of fatty acyl CoA derivatives is a *sine qua non* for the study of fatty acid oxidation in systems of soluble enzymes It is, therefore, important to review the methods by which such derivatives can be prepared The available methods of synthesis can be divided into two categories. (1) enzymic synthesis and (2) chemical synthesis

In our laboratory we have concentrated almost exclusively on the application of specific enzymes for the preparation of acyl derivatives of CoA The acetic thiokinase (*22–24*) which catalyzes the ATP-dependent formation of acetyl and propionyl CoA from the corresponding acids and CoASH according to Eq (11) is eminently suited for synthetic purposes



$$CH_3COOH + ATP + CoASH \rightarrow CH_3COSCoA + AMP + PP \quad (11)$$

In presence of an excess of acetate and ATP, CoASH is almost quantitatively esterified The acyl CoA's can be separated from the other com-

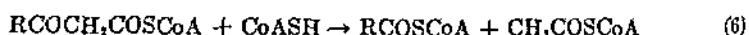
[3] According to a recent report from Lynen's laboratory (*13*) the deacylation of *S*-acetoacetyl CoA is not a direct process but the sum of two separate reactions first, the condensation of acetyl CoA with acetoacetyl CoA to form β-hydroxy-β-methyl glutaryl CoA (HMG CoA) [cf refs (*14*, *15*)], and then the cleavage of the latter to acetyl CoA and acetoacetate [cf Bachhawat *et al* (*16*)]

A second dehydrogenation then takes place—this time at the β-carbon atom—which is catalyzed by β-hydroxyacyl dehydrogenase with DPN as specific electron acceptor:



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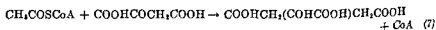
Finally, β-ketoacyl thiolase cleaves the β-ketoacyl derivative according to the following equation:



$$RCOCH_2COSCoA + CoASH \rightarrow RCOSCoA + CH_3COSCoA \quad (6)$$

The sum of Eqs (2–6) is in fact equivalent to (1) except that RCOOH and the C_2 unit in the latter equation are represented as the free fatty acids rather than as their esters of coenzyme A. At the completion of each sequence represented by Eqs. (2–6) the fatty acid is degraded by two carbon atoms A new fatty acid is thus generated which can then in turn undergo a repeat of the degradation process.

Acetyl CoA, the C_2 unit which is the end product of each degradative sequence condenses with oxalacetate to form citrate in presence of a specific condensing enzyme which has been thoroughly studied by the *Ochoa school (12).*



$$CH_3COSCoA + COOHCOCH_2COOH \rightarrow COOHCH_2(COHCOOH)CH_2COOH + CoA \quad (7)$$

Since citrate is oxidized eventually to CO_2 and water by way of the citric acid cycle, the reaction described by Eq (7) may be looked upon as the one linking the two key mitochondrial cycles—the fatty acid and the citric acid cycles

The six basic enzymic reactions which make up the fatty acid oxidation sequence are thus (1) acylation of CoA by the fatty acid. (2) α,β-dehydrogenation; (3) hydration of the α,β-unsaturated derivative; (4) *β-oxidation of the β-hydroxyacyl CoA to the corresponding* β-ketoacyl CoA; (5) thiolytic cleavage of the β-ketoacyl CoA to a fatty acyl CoA and acetyl CoA, and finally (6) condensation of acetyl CoA and oxalacetate to form citrate

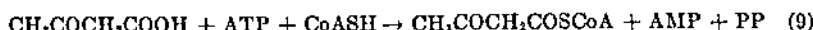
There are a few additional enzymic reactions which have to be considered in the context of fatty acid oxidation In liver *mitochondria* fatty acid oxidation proceeds partly to completion, i e, to CO_2 and water, *while the rest goes no further than to the* stage of acetoacetate The accumulation of acetoacetate as an end product of fatty acid oxidation in liver is the consequence of the presence of an enzyme system which in

effect catalyzes the deacylation of *S*-acetoacetyl CoA to acetoacetate and CoASH[1] (*17–19*).



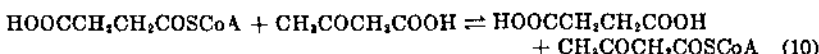
$$CH_3COCH_2COSCoA + H_2O \rightarrow CH_3COCH_2COOH + CoASH \quad (8)$$

Since the deacylation reaction is irreversible and furthermore, since liver is deficient or lacking in the enzyme which catalyzes the acylation of CoA by acetoacetate (*10*),



$$CH_3COCH_2COOH + ATP + CoASH \rightarrow CH_3COCH_2COSCoA + AMP + PP \quad (9)$$

acetoacetate accumulates in liver and not in other tissues It is formed in other tissues, but does not accumulate because of one or both of two active processes (1) the ATP-catalyzed conversion to the acyl CoA as in Eq (9), (2) the succinyl CoA-catalyzed conversion of acetoacetate to the acyl CoA by thiophorase—an enzyme which labilizes the thioester bond of acyl CoA esters and catalyzes the replacement of succinyl by acetoacetate or acetoacetyl by succinate (*20, 21*), Eq (10)

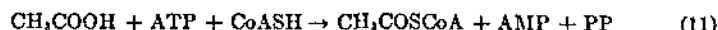


$$HOOCCH_2CH_2COSCoA + CH_3COCH_2COOH \rightleftharpoons HOOCCH_2CH_2COOH + CH_3COCH_2COSCoA \quad (10)$$

C Preparation of Acyl Coenzyme A Derivatives

The availability of fatty acyl CoA derivatives is a *sine qua non* for the study of fatty acid oxidation in systems of soluble enzymes It is, therefore, important to review the methods by which such derivatives can be prepared The available methods of synthesis can be divided into two categories (1) enzymic synthesis and (2) chemical synthesis

In our laboratory we have concentrated almost exclusively on the application of specific enzymes for the preparation of acyl derivatives of CoA The acetic thiokinase (*22–24*) which catalyzes the ATP-dependent formation of acetyl and propionyl CoA from the corresponding acids and CoASH according to Eq (11) is eminently suited for synthetic purposes



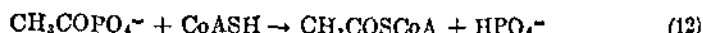
$$CH_3COOH + ATP + CoASH \rightarrow CH_3COSCoA + AMP + PP \quad (11)$$

In presence of an excess of acetate and ATP, CoASH is almost quantitatively esterified The acyl CoA's can be separated from the other com-

[1] *According to a recent report from Lynen's laboratory* (*13*) the deacylation of *S*-acetoacetyl CoA is not a direct process but the sum of two separate reactions first, the condensation of acetyl CoA with acetoacetyl CoA to form β-hydroxy-β-methyl glutaryl CoA (HMG CoA) [cf refs (*14, 15*)], and then the cleavage of the latter to acetyl CoA and acetoacetate [cf Bachhawat *et al* (*16*)]

ponents of the incubation mixture by extraction with phenol solution (*22*, *25*) or by chromatographic means (*26*).

Stadtman (*26*, *27*) has prepared acetyl CoA from acetyl phosphate and CoASH with the aid of transacetylase—an enzyme isolated from *Clostridium kluyveri* which catalyzes the following reversible reaction:



$$CH_3COPO_4^{=} + CoASH \rightarrow CH_3COSCoA + HPO_4^{=} \quad (12)$$

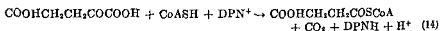
With an excess of acetyl phosphate the conversion of CoASH to acetyl CoA is essentially quantitative

An oxidative enzyme—aldehyde dehydrogenase—obtained from *C. kluyveri* can also be applied to the synthesis of acetyl CoA This enzyme which has been studied by Stadtman (*27*) and Burton (*28*) catalyzes the following reversible reaction:



$$CH_3CHO + CoASH + DPN^+ \rightarrow CH_3COSCoA + DPNH + H^+ \quad (13)$$

This is an oxidative acylation of CoASH which is analogous to the oxidative succinylation of CoASH by α-ketoglutarate—a reaction described by Sanadi and Littlefield (*29a*) and Kaufman *et al.* (*29b*):

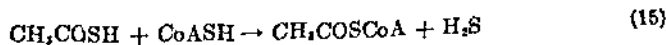


$$COOHCH_2CH_2COCOOH + CoASH + DPN^+ \rightarrow COOHCH_2CH_2COSCoA + CO_2 + DPNH + H^+ \quad (14)$$

The enzymic acylation of CoASH by higher fatty acids and fatty acid derivatives is carried out with the general fatty acid thiokinase of Mahler *et al* (*10*) and the long-chain fatty acid thiokinase of Kornberg and Pricer (*30*) These two enzymes carry out the synthesis according to the reaction described in Eq (2) The Mahler enzyme is remarkably versatile. It can be used successfully for the synthesis of short-chain acyl CoA's from C_4 to C_{10} and of α,β- or β,γ-unsaturated acyl CoA's and β-hydroxyacyl CoA's covering the same range of chain length

Beinert (*31*) has prepared β-ketoacyl CoA derivatives from α,β-unsaturated acids in a three-step enzymic synthesis involving the combined action of thiokinase, Eq (2), enoyl hydrase, Eq (4), and β-hydroxyacyl dehydrogenase, Eq. (5). Coenzyme A is acylated by the α,β-unsaturated fatty acid The acyl ester after hydration is oxidized to the corresponding β-ketoacyl CoA derivative The oxidative reaction has to be carried out at pH 9 in presence of Mg^{++} Under these conditions the equilibrium point is shifted strongly in favor of formation of the β-ketoacyl CoA

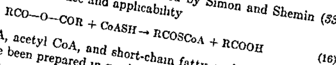
A variety of methods are now available for the chemical synthesis of acyl CoA esters. The following method of Wilson (*32*) was the first successful chemical synthesis to be reported in the literature:



$$CH_3COSH + CoASH \rightarrow CH_3COSCoA + H_2S \quad (15)$$

Although this exchange reaction does not enjoy a favorable equilibrium the method is still in general use.

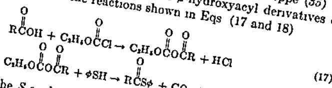
The acid anhydride method introduced by Simon and Shemin (33) has found wide acceptance and applicability.



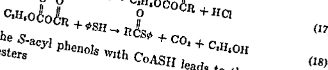
$$RCO{-}O{-}COR + CoASH \rightarrow RCOSCoA + RCOOH \quad (16)$$

Succinyl CoA, acetyl CoA, and short-chain fatty acyl CoA esters from C_4 to C_8 have been prepared in good yield by this method. The reaction is carried out in aqueous medium in presence of bicarbonate. Seubert (34) reported a variant of this method in which the acid anhydride is replaced by the acid chloride.

The interaction of ethylchlorocarbonate with fatty acids yields ethylacylcarbonates which can acylate thiophenol in good yield. This method has been successfully applied by Wieland and Koeppe (35) to the synthesis of *S*-acetyl, *S*-lactyl, and *S*-β-hydroxyacyl derivatives of thiophenol according to the reactions shown in Eqs. (17 and 18).

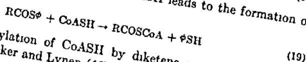


$$R\overset{O}{\overset{\|}{C}}OH + C_2H_5O\overset{O}{\overset{\|}{C}}Cl \rightarrow C_2H_5O\overset{O}{\overset{\|}{C}}O\overset{O}{\overset{\|}{C}}R + HCl \quad (17)$$



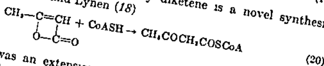
$$C_2H_5O\overset{O}{\overset{\|}{C}}O\overset{O}{\overset{\|}{C}}R + \phi SH \rightarrow R\overset{O}{\overset{\|}{C}}S\phi + CO_2 + C_2H_5OH \quad (18)$$

Exchange of the *S*-acyl phenols with CoASH leads to the formation of the acyl CoA esters.



$$RCOS\phi + CoASH \rightarrow RCOSCoA + \phi SH \quad (19)$$

The acetoacetylation of CoASH by diketene is a novel synthesis introduced by Decker and Lynen (18).



$$CH_2{-}C{=}CH + CoASH \rightarrow CH_3COCH_2COSCoA \quad (20)$$
(ring: $O{-}C{=}O$)

This method was an extension of Bentley's discovery that inorganic phosphate can be acetylated by ketene to form acetyl phosphate (36).

The synthetic methods involve a complication which does not usually apply to the enzymic synthesis of coenzyme A esters. Since the commercial preparations of coenzyme A which are the starting points for synthesis may contain some 5% by weight of glutathione, the end products of the chemical synthesis contain the *S*-acyl esters of both coenzyme A and glutathione. The SH-group of the tripeptide behaves in exactly the same fashion as that of CoA towards the various acylating reagents described above. This difficulty does not apply to the enzymic synthesis since the enzymes can readily distinguish between CoASH and

thione as acyl acceptors (*10*). The alternatives are either to remove glutathione completely from the preparation of CoASH by chromatographic procedures—a rather laborious process—or to change over to an enzymic synthesis

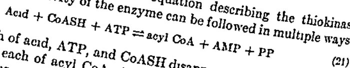
In general the purity of the acyl CoA esters synthesized is usually no higher than that of the starting coenzyme A. The purity of the commercial preparation now available (Pabst) varies from 60 to 75% Undoubtedly some of the impurity is dephospho-CoA which is either a degraded form of CoA or a precursor of CoA in which one of the three phosphate groups (located in the 3′-position of ribose) has been split off (*37*).

D. The Properties of Enzymes Concerned in the Fatty Acid Oxidation Cycle

1 *Acid Thiokinase*

Three separate thiokinases have been described to date: (1) an enzyme obtained from heart mitochondria which is active on acetic and propionic acids (*22–24*), (2) an enzyme from beef liver mitochondria which covers the range from C_4 to C_{12} (*10*), and (3) an enzyme associated with submitochondrial particles of guinea pig liver which is active on the higher fatty acids from C_8 to C_{22} (*30*) The acetic thiokinase does not show any significant activity toward substituted fatty acids (e g , lactic and glycollic acids) whereas the medium-chain thiokinase is active on a wide variety of acids including the α,β- and β,γ-unsaturated acids, the β-hydroxy-acids of both the D- and L-configurational series, phenyl-fatty acids as well as fatty acids linked to other ring systems (*10*) The specificity of the long-chain thiokinase with respect to substituted fatty acids has yet to be fully explored

It is obvious from the general equation describing the thiokinase reaction that the activity of the enzyme can be followed in multiple ways.



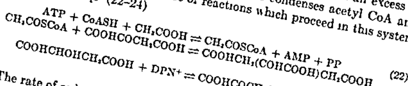
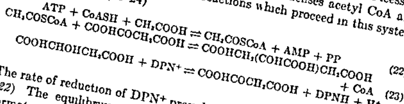
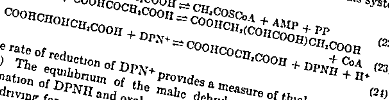
$$\text{Acid} + \text{CoASH} + \text{ATP} \rightleftharpoons \text{acyl CoA} + \text{AMP} + \text{PP} \qquad (21)$$

One mole each of acid, ATP, and CoASH disappears during the synthesis and one mole each of acyl CoA, AMP, and inorganic pyrophosphate is formed. The disappearance of the thiol group of CoASH can be readily followed by the delicate nitroprusside method of Grunert and Phillips (*38*) Mahler *et al* have used this method for the assay of beef liver thiokinase (*10*) Assay procedures based on the appearance of inorganic pyrophosphate (*39*) or the disappearance of ATP are also practical. Stadtman (*27*) and Lynen (*40*) recognized that acyl thioesters of CoA show a band at 233 mμ which is characteristic of the thioester bond (*41*) Stadtman has developed a spectrophotometric assay of the transacetylase reaction [cf Eq (12)] which is based on the appearance or disappearance

of the 233 mμ band depending whether the reaction is carried out in one or other direction.

A method which featured prominently in some of the early work thiokinases depends upon the principle that acyl thioesters of coenzyme (in fact all anhydrides) react with hydroxylamine to form acyl hydr amates which are then readily estimated by the colorimetric procedu of Lipmann and Tuttle (*42*) (complexing with ferric chloride). Th enzymic reaction is carried out in the presence of high levels of hydrox ylamine which presumably traps the acyl CoA ester as it is formed (*22, 23*) This method of assay is not entirely reliable since high levels of hydroxylamine may inhibit thiokinases (*10, 22*) Furthermore, there is some indication that an enzyme additional to thiokinase may be required for the interaction of NH_2OH with the acyl CoA at least under the conditions of the assay (*22*)

Perhaps the most satisfactory assay system for acetic thiokinase is that in which thiokinase in limiting amount is linked with an excess of malic dehydrogenase and the enzyme which condenses acetyl CoA and oxalacetate (*43*) The sequence of reactions which proceed in this system is shown in Eqs (22–24)

$$ATP + CoASH + CH_3COOH \rightleftharpoons CH_3COSCoA + AMP + PP \quad (22)$$

$$CH_3COSCoA + COOHCOCH_2COOH \rightleftharpoons COOHCH_2(COHCOOH)CH_2COOH + CoA \quad (23)$$

$$COOHCHOHCH_2COOH + DPN^+ \rightleftharpoons COOHCOCH_2COOH + DPNH + H^+ \quad (24)$$

The rate of reduction of DPN^+ provides a measure of thiokinase activity (*22*) The equilibrium of the malic dehydrogenase is unfavorable to formation of DPNH and oxalacetate. Thus acyl CoA formation becomes the driving force for continuous reduction of DPN since the product of oxidation of malate is condensed with acyl CoA as quickly as formed

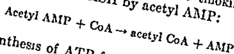
The thiokinase reaction has been shown to be reversible Mahler *et al* (*10*) have determined the K_{eq} [defined by Eq (25)] for the beef liver thiokinase system

$$K_{eq} = \frac{[AMP^-][PP^=][RCOSCoA]}{[ATP^=][CoASH][RCOO^-]} \quad (25)$$

At 38°, and pH 8.0, with heptanoic acid as RCOOH, the value for K_{eq} is 1.11

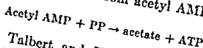
The mechanism of the thiokinase reaction has been the subject of intense interest in recent years although the final work has yet to be written Pyrophosphoryl CoA (*44, 45*) was the first intermediate to be postulated on the basis of some isotope data which were later shown to be ambiguous (*46*) Evidence was presented to support the hypothesis

that enzyme-CoA and enzyme-AMP complexes were intermediate the reaction (*46*) More recently Berg (*47*) synthesized acetyl-AMP compound in which the acyl group is linked to the phosphate of AMP an ester bond He demonstrated that acetic thiokinase of yeast catalyze the acetylation of CoASH by acetyl AMP:



$$\text{Acetyl AMP} + \text{CoA} \rightarrow \text{acetyl CoA} + \text{AMP} \quad (26)$$

as well as the synthesis of ATP from acetyl AMP and inorganic pyrophosphate:



$$\text{Acetyl AMP} + \text{PP} \rightarrow \text{acetate} + \text{ATP} \quad (27)$$

Peng (*48*) and Talbert and Huennekens (*49*) have made similar observations with the fatty acid thiokinase of beef liver. Butyryl AMP was shown to be capable of forming either ATP and butyrate or butyryl CoA and AMP. Acetyl AMP and butyryl AMP thus satisfy two important criteria for a true intermediate However, one further condition is not met Under no conditions yet found can acetyl AMP or butyryl AMP be isolated as an intermediate in the thiokinase reaction [see Eq. (21)] whether the reaction is proceeding in the left to right direction or in the reverse direction Thus if a compound corresponding to acetyl AMP is indeed formed in the course of the thiokinase reaction, it can only exist in a bound form

Magnesium ions are an absolute requirement for the activity of all thiokinases described thus far This fact has been the point of departure for an hypothesis (*50*) as to mechanism suggested by Ingraham and Green which takes the following form Magnesium forms a chelate complex with enzyme, ATP, and CoASH. Acetate then reacts directly with the magnesium and enzyme-bound ATP and displaces inorganic pyrophosphate Again within the complex the acetyl group is transferred to CoASH, and then the complex dissociates into acetyl CoA, adenosine monophosphate, and inorganic pyrophosphate The sequence of events is summarized schematically in Fig 1 The thermodynamic advantage of the magnesium chelate is that the reactants are gathered together first, and then interaction takes place This means that the entropy term falls out, and the activation energy becomes correspondingly reduced

2. *Fatty Acyl Dehydrogenases*

Beef and pig liver mitochondria have been shown to contain, respectively, two and three separate enzymes, all flavoproteins, which collaborate in catalyzing the first oxidative step of the fatty acid oxidation cycle, i.e., the oxidation of a saturated fatty acyl CoA derivative to the corresponding α,β-unsaturated derivative. The separation of these

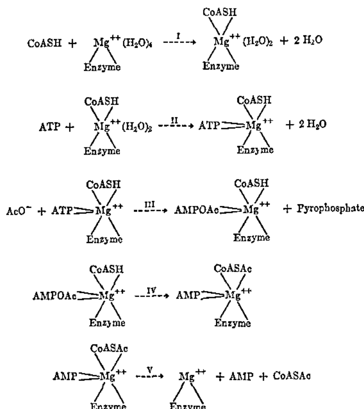


FIG 1 Structural forms of the hypothetical intermediates in the thiokinase system [from Ingraham and Green (*50*)]

flavoproteins from one another and their preparation in a highly purified state has now been accomplished in our laboratory (*51–53*) A green-colored dehydrogenase (G) is specific for short-chain fatty acyl CoA esters (cf Fig 2) (*51*, *54*) The other two flavoproteins, Y_1 (*52*) and Y_2 (*53*) show no color anomalies Y_1 is specialized for the oxidation of the medium-chain acyl esters while Y_2 is adapted to the oxidation of the long-chain esters

In attempting to decide which substrates are acted upon by which flavoprotein enzyme under physiological conditions it is not enough to consider merely the optimum velocity which an enzyme can attain in presence of an excess of a given substrate especially if the substrate concentration exceeds the upper limit of the physiological concentration

range. The concentration of substrate at which one-half the maximal velocity is attained (i e., the Michaelis constant, K_m) is inversely related to the affinity of the enzyme for a given substrate and serves as a reliable guide for the pairing of substrate and enzyme For example, pig liver Y_1 is active on fatty acyl CoA esters from C_4 to C_{16} but shows a higher

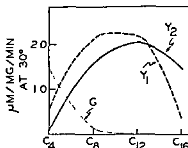


FIG 2 Specificity of fatty acyl dehydrogenases (G, Y_1, and Y_2) from pig liver for acyl CoA substrates of different chain length Ordinate, specific activity of enzyme under standard assay conditions (*52*), abscissa, number of carbons in fatty acid moiety of substrate

affinity for the C_8 ester than for the C_4 or C_{16} ester (*55*) (cf. Table I) The affinity declines as the chain length of the ester gets shorter or longer Given a mixture of C_4 and C_8 esters in equimolar concentrations the longer chain ester would take precedence whereas in a mixture of C_8 and C_{16} esters the shorter chain ester would take precedence. The Michaelis

TABLE I
K_m VALUES OF ACYL DEHYDROGENASES IN RELATION TO CHAIN LENGTH OF SUBSTRATE (*55*)

Acyl CoA Substrate	Acyl Dehydrogenase	$K_m \times 10^6$ (moles)
C_4	G	2 2
C_4	Y_1	40 0
C_8	Y_1	0 9
C_{16}	Y_1	3 4
C_8	Y_2	1 2
C_{16}	Y_2	1 6

constant K_m for the most effective substrates of Y_1 is about $1 \times 10^{-6} M$ A K_m of the same order of magnitude applies to the other acyl dehydrogenases (*55*).

Some pertinent data on the composition, probable molecular weight, and spectral characteristics of G, Y_1, and Y_2 from pig liver mitochondria

are summarized in Table II Beinert and his colleagues have found clear evidence of two analogous enzymes in beef and pig heart muscle, viz, G and Y Crane *et al* (*52*) have found that a flavoprotein can be isolated from beef liver which has several properties in common with Y_1 of pig liver but is not identical There may thus be species differences between enzymes with similar or identical metabolic function

The green color of G was first attributed to the presence of copper, but this could not be substantiated It now appears that some as yet unknown group in the protein forms a complex with the flavin prosthetic

TABLE II
PROPERTIES OF ACYL DEHYDROGENASES FROM PIG LIVER MITOCHONDRIA

Property	G[a]	Y_1 (*52*)	Y_2 (*53*)
Absorption maxima	267, 357, 426, 710	275, 370, 447	275, 365, 442
Ratio of absorption maxima	8 1 0 82 1 0 0 34	6 70 0 72 1 0	8 1 0 74 1 0
Riboflavin content	0 60%	0 42%	0 42%
Minimum molecular weight (riboflavin basis)	approx 70,000	85,000–95,000	85,000–95,000
Molecular weight (from sedimentation data)	140,000–200,000	140,000–200,000	140,000–200,000

[a] Unpublished

group of the enzyme, and this internal complex is responsible for the green color of the enzyme. Under appropriate conditions the complex responsible for the green color can be decomposed and then the enzyme regains the characteristic yellow color of classic flavoproteins (*56*)

The product of the oxidation is presumed to be the *trans*-α,β-derivative for the following reasons (1) When enoyl hydrase and β-hydroxyacyl dehydrogenase—two enzymes which follow one another in the fatty acid oxidation sequence—are allowed to act on the product of the first oxidation, complete transformation of the product is observed (*52*) Such a result would be possible only if the unsaturated product of the first oxidation were in the *trans*-form. On that basis the *cis*-form can be excluded The question of the optical and geometrical specificity of enoyl hydrase will be considered fully in a later section. The possibility of an equilibrium between the *cis*- and *trans*-forms is one to be considered in studies carried out with crude extracts However, the complication does not apply to studies with highly purified enzymes of the fatty acid oxidation sequence (2) Since G can oxidize isobutyryl CoA which has no possibility of β,γ-dehydrogenation (*52*), at least for this substrate, α,β-dehydrogenation is the only choice open. Thus these two lines of

evidence suggest that the product of oxidation of fatty acyl coenzyme A esters by the acyl dehydrogenases is the *trans*-α,β-unsaturated ester

The binding of substrate by the acyl dehydrogenases is essentially irreversible in the sense that the complex is not dissociable by dialysis or precipitation of the enzyme However, a molecule of substrate which is bound can be displaced by another molecule of substrate. In this way the possibility of turnover is achieved. Instead of the product of oxidation dissociating from the enzyme it undergoes displacement (*57*)

The three flavoprotein dehydrogenases mentioned above catalyze the oxidation of their respective substrates by phenazine methosulfate or quinones but not by ferricyanide, 2,6-dichlorophenol-indophenol or cytochrome *c* However, when any one of these dehydrogenases is supplemented with a specific flavoprotein (*58*) obtained from pig liver mitochondria, then this combination of two enzymes can catalyze the oxidation of substrate with all the acceptors mentioned above Furthermore the rate of oxidation with phenazine methosulfate and quinones which was relatively slow with the acyl dehydrogenase alone is greatly increased in presence of the two-enzyme combination The second flavoprotein which has been named the electron transferring flavoprotein (ETF) undergoes a cycle of reduction by the reduced acyl dehydrogenase and then of reoxidation by the terminal electron acceptor (*58*) This is a unique instance in which a special flavoprotein has to intervene in the transfer of electrons from the reduced primary dehydrogenase to the electron acceptor. ETF must therefore be classified as a dehydrogenase whose substrate is a reduced acyl dehydrogenase.

The behavior of the flavin prosthetic group of the three acyl dehydrogenases is atypical Ordinarily flavoproteins in their reduced state are rapidly autoxidizable in air But the acyl dehydrogenases when enzymically reduced are stable almost indefinitely in air The prosthetic flavins appear to be inaccessible to molecular oxygen and for that matter to a whole group of electron acceptors which ordinarily oxidize reduced flavoproteins with high velocity ETF is presumably designed structurally to tap electrons as it were, from the reduced forms of these well-screened flavin prosthetic groups The meaning of this immobilization of the prosthetic flavin as well as the molecular basis for its accomplishment are both still obscure

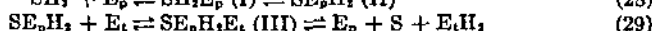
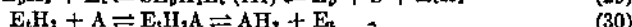
ETF is a flavoprotein of about 80,000 molecular weight (*58*) Per mole of enzyme there is one mole of flavin in the form of flavin adenine dinucleotide Metal analysis has failed to disclose the presence of any metal in ETF in sufficient amount to implicate this flavoprotein as a metalloflavoprotein However, the absorption spectrum of ETF has at least two anomalous features The main absorption band in the visible has a

maximum at 437.5 mμ There is an additional minor peak at 460 mμ which shows up as a distinct shoulder on the main band Furthermore, the $E_{270\ m\mu}$ $E_{437.5\ m\mu}$ ratio (protein flavin ratio) is the lowest recorded for any flavoprotein

Hauge (*55*) has subjected the system—substrate, acyl dehydrogenase, ETF, electron acceptor—to a kinetic analysis He has pointed out that the Michaelis constants and the turnover numbers for the acyl dehydrogenase or for ETF cannot be arrived at from measurements of the over-all reaction—at least not by the conventional Michaelis-Menten system of equations The apparent Michaelis constants and turnover numbers differ from the true constants by unknown factors, and the former refer only to the total substrate-enzyme-acceptor system

The velocity of interaction of the two flavoproteins with one another is remarkably high considering the complexity of the reacting species The observed turnover number of 280 per minute at 38° for ETF is probably minimal, and the true turnover number may be several-fold higher The affinity of ETF for the acyl dehydrogenase is also very high The apparent Michaelis constant has a value of about 3×10^{-7} *M*

According to Hauge (*55*) the sequence of molecular events which take place during the dehydrogenation of acyl coenzyme A esters may be represented by the reactions shown in Eqs (28–30) Let E_p represent the oxidized form of the primary dehydrogenase, E_pH_2 the reduced form of this enzyme, SH_2 the reduced form of the substrate, S the oxidized form, E_t the oxidized form of the electron transferring flavoprotein, E_tH_2 the corresponding reduced form, and A and AH_2 the oxidized and reduced forms, respectively, of some electron acceptor


$$SH_2 + E_p \rightleftharpoons SH_2E_p\ (I) \rightleftharpoons SE_pH_2\ (II) \qquad (28)$$

$$SE_pH_2 + E_t \rightleftharpoons SE_pH_2E_t\ (III) \rightleftharpoons E_p + S + E_tH_2 \qquad (29)$$

$$E_tH_2 + A \rightleftharpoons E_tH_2A \rightleftharpoons AH_2 + E_t \qquad (30)$$

These equations express the following notions (1) substrate and dehydrogenase form a complex (I) which by intramolecular oxido-reduction emerges as a complex of oxidized substrate and reduced enzyme (II) (2) ETF can react with (II) to form yet another complex (III) in which substrate, acyl dehydrogenase and ETF are all linked together After intramolecular oxido-reduction the complex dissociates into reduced ETF, and oxidized substrate and acyl dehydrogenase. (3) Finally, reduced ETF undergoes oxidation by the terminal electron acceptor (A). The product of the oxidation (α,β-unsaturated acyl CoA) has a higher affinity for the acyl dehydrogenase than the substrate of the oxidation (fatty acyl CoA). This discrepancy in affinities accounts for the serious competitive inhibition exerted by the product of the oxidation.

since octanoyl CoA effects more pronounced bleaching than butyryl or palmityl CoA at the same concentration. (Compare these relationships with the K_m values in Table I)

Seubert and Lynen *(63)* have described an enzyme preparation from sheep liver which catalyzes the oxidation of leuco-safranine by *S*-crotonyl-*N*-acetylthioethanolamine though much more slowly than by crotonyl CoA *Apparently the acyl dehydrogenases can react with S-acyl compounds* which are less complex than esters of coenzyme A. Similarly the

TABLE III
REDUCTION OF THE Y_1 ACYL DEHYDROGENASE *(59)*[a]

Acyl CoA Substrate (mμmoles)	C_4	C_8	C_{16}
1 2	0 018	0 088	0 044
12	0 085	0 197	0.085
200	0 145	0 200	0 085

[a] The data presented are the decrements in absorbance at 447 mμ upon addition of different amounts of C_4, C_8, and C_{16} acyl CoA substrates to a fixed quantity of completely oxidized Y_1 acyl dehydrogenase. Dithionite chemically reduced the same quantity of enzyme to the extent of 0 254 absorbancy units

acyl dehydrogenases can catalyze the oxidation of *S*-butyryl-*N*-acetylthioethanolamine by appropriate electron acceptors Lynen *et al* *(45, 64)* and Stern *(65)* have studied these synthetic substrates in considerable detail not only with respect to acyl dehydrogenase but also with respect to the other enzymes of the fatty acid oxidation cycle

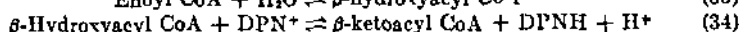
3 *Enoyl Hydrase*

Thus far only one enzyme has been isolated from mitochondria which is concerned in the hydration of α,β-unsaturated acyl coenzyme A esters This enzyme was first isolated by Mahler and Wakil *(66, 67)* in 1953 from beef liver mitochondria More recently Stern *et al.* *(68a)* have prepared the enzyme from whole beef liver and have succeeded in obtaining it in homogeneous and crystalline state The first reports of Stern and Del Campillo *(68b)* gave the impression that their preparation of enoyl hydrase (referred to as crotonase by these authors) had a more restricted range of substrate activity than the Mahler-Wakil preparation, but in the light of more recent work it would appear that there is no basis for assuming that the same enzyme is not involved in both cases.

Enoyl hydrase of beef liver has a molecular weight of about 210,000 *(68a)*. One mole of the crystallized enzyme catalyzes the hydration of 730,000 moles of crotonyl CoA per minute at pH 7.5 and 25°. At pH 9.4 the turnover number is 1,400,000.

There are two methods available for assay of enoyl hydrase The first method (*67*) requires the presence of an additional enzyme, viz β-hydroxyacyl dehydrogenase (used in excess) and depends upon the following two reactions


Enoyl CoA + $H_2O \rightleftarrows$ β-hydroxyacyl CoA (33)


β-Hydroxyacyl CoA + $DPN^+ \rightleftarrows$ β-ketoacyl CoA + DPNH + H^+ (34)

The reaction may be followed continuously by measuring the rate of reduction of DPN^+ to DPNH spectrophotometrically Since both reactions in the two-step sequence are equilibrium reactions and since at pH 7 0 the equilibria are not favorable for the left to right reaction, a device has to be found for shifting the equilibrium point This is accomplished by conducting the over-all assay at pH 9 in presence of Mg^{++}. At this pH, reduction of DPN^+ to DPNH is essentially complete Furthermore, the formation of a complex between β-ketoacyl CoA and Mg^{++} accentuates even further the left to right bias of the reaction The limitation of the above assay is that unless the product of the first reaction catalyzed by hydrase can serve as the substrate for the dehydrogenase catalyzing the second reaction, the assay is not a valid measure of hydrase activity It was, in fact, this particular difficulty which obscured for several years one of the basic features of hydrase activity

The second assay (*68a*) depends upon the fact that α,β-unsaturated acyl CoA esters have two characteristic absorption bands in the ultraviolet—at 263 and 224 mμ respectively The free acids, the β,γ-unsaturated acyl CoA esters and the β-hydroxyacyl CoA esters do not show these bands The conjugation between the double bond in β,α-position, and the carboxyl group is presumably responsible for these bands The rate of hydration or the rate of dehydration of substrates by enoyl hydrase can be followed spectrophotometrically by measuring the rate of change in absorption at either 263 mμ or 224 mμ

The turnover number of enoyl hydrase is several-fold higher in the direct one-step spectrophotometric assay than in the two-step enzymic assay It is, therefore, not justifiable to compare the relative activities of two preparations of enoyl hydrase when the two preparations have not been assayed by the same method When the specific activity of the crystalline preparation of Stern is compared with the activity of the purified Mahler-Wakil preparation of enoyl hydrase under the same assay conditions the differences in activity are slight.

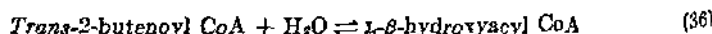
Enoyl hydrase can hydrate both the *cis*- and *trans*-forms of 2-butenoyl-*S*-CoA which are referred to generally as isocrotonyl and crotonyl CoA respectively (*69, 70*) The *trans*-form is hydrated 2.5 times faster than

the *cis*-form under comparable conditions When the *cis*-form is hydrated, the product is D-β-hydroxyacyl CoA; when the *trans*-form is hydrated, the product is L-β-hydroxyacyl CoA (*70*). There appears to be no interconversion of *cis*- and *trans*- in presence of the purified enzyme nor racemization of the D- and L-β-hydroxyacyl CoA enantiomorphs Stern *et al.* (*69*) have reported that *cis*- and *trans*-enoyl CoA esters yield on hydration the same optical isomer viz. the L-form However, since the preparations of esters which they tested were of commercial origin it is difficult to evaluate their conclusion that the hydration of *cis*- and *trans*-isomers leads to the same optical enantiomorph. The complete separation of *cis*- and *trans*-butenoyl CoA esters is a difficult technical accomplishment because of the ease with which the *cis*-esters are isomerized.

The equilibrium value K defined by the equation:

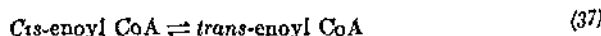
$$K = \frac{[\beta\text{-hydroxyacyl CoA}]}{[\text{enoyl CoA}][H_2O]} \quad (35)$$

for the reaction·



$$\textit{Trans}\text{-2-butenoyl CoA} + H_2O \rightleftharpoons \text{L-}\beta\text{-hydroxyacyl CoA} \quad (36)$$

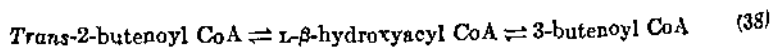
has been measured by Wakil and Mahler (*67*) at 25° and found to be $3\,6 \times 10^{-2}$ mole^{-1}. The K value for the corresponding equilibrium with *cis*-2-butenoyl CoA is $7\,5 \times 10^{-2}$ mole^{-1} at 25° The difference in the values of the two respective equilibrium constants provides a measure of the ΔF for the reaction



$$\textit{Cis}\text{-enoyl CoA} \rightleftharpoons \textit{trans}\text{-enoyl CoA} \quad (37)$$

The free energy change of isomerization calculated from the difference in free energies of the two respective hydration reactions is about 400 calories

Enoyl hydrase catalyzes the hydration of both 2-butenoyl-*S*-CoA and 3-butenoyl-*S*-CoA (vinylacetyl CoA) (*67*, *71*) The product of hydration of the β,γ-unsaturated ester is L-β-hydroxyacyl CoA Since the product formed by the hydration of the *trans*-α,β- and the β,γ-butenoyl CoA esters respectively is identical, it would follow that an equilibrium must be established between these two esters.



$$\textit{Trans}\text{-2-butenoyl CoA} \rightleftharpoons \text{L-}\beta\text{-hydroxyacyl CoA} \rightleftharpoons \text{3-butenoyl CoA} \quad (38)$$

Stern and Del Campillo (*71*) in fact have adduced spectrophotometric evidence that crotonyl CoA is formed to the extent of 8 to 10% when enoyl hydrase is added to vinylacetyl CoA Thus, three molecular species are in equilibrium with one another in presence of hydrase

According to Wakil and Mahler (*67*) enoyl hydrase acts on all β-hydroxyacyl CoA or enoyl CoA esters from C_4 to at least C_{12} Apparently the enzyme is unspecific for the chain length of the acyl ester Stern and Del Campillo (*71*) claim that there is an inverse relation between velocity and chain length, but the data which they present to support this generalization are not complete They have reported that the C_9-β-hydroxyacyl CoA ester is dehydrated by the crystalline enzyme but have not tested higher homologs

Enoyl hydrase is able to catalyze the hydration of sorbyl CoA which has a double bond in 1-2 and 3-4 positions It must be inferred that only the 1-2 double bond is hydrated and that the product is L-2-hydroxy-4-hexenoyl CoA This in turn can be oxidized by the β-hydroxyacyl dehydrogenase. After one complete turn of the fatty acid oxidation cycle, crotonyl CoA is formed and then the cycle of hydration, oxidation and cleavage can be repeated

Hydrase has no action on 4-enoyl CoA derivatives such as 4-pentenoyl-*S*-CoA or on acrylyl-*S*-CoA (*71*) It is also inactive on any free acids tested whether *cis*- or *trans*- (*67*)

Unlike the acyl dehydrogenase, enoyl hydrase is fairly specific for the thiol portion of the acyl thioester Coenzyme A cannot be replaced by glutathione or *N*-acetyl thioethanolamine *S*-pantotheine enoyl esters are indeed hydrated enzymically, but the rate is vanishingly small compared to that of the corresponding esters of CoA (*65*)

4 β-*Hydroxyacyl Dehydrogenase*

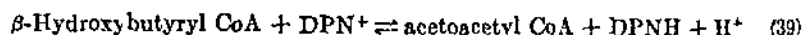
Wakil *et al* (*72*) have isolated from beef liver mitochondria a soluble enzyme which catalyzes the oxidation of β-hydroxyacyl CoA esters from C_4 to at least C_{12} The available evidence is consistent with the assumption that all these oxidations are catalyzed by the same enzyme DPN is the specific electron acceptor for the apodehydrogenase

The enzyme can be assayed by either of two methods. The first depends upon the fact that the dehydrogenation reaction proceeds essentially to completion at about pH 9 in presence of Mg^{++} Thus the rate of DPN reduction can serve as a convenient measure of the activity of the enzyme The second method depends upon the fact that the product of the reaction, viz , the β-ketoacyl CoA has a characteristic band at 303 mμ (*5, 64*) Thus the rate of increase in absorption at 303 mμ during the dehydrogenation reaction provides a measure of the activity of the enzyme The dehydrogenase is the only enzyme required in either of these two alternative assays

Lynen and his co-workers (*5, 64*) have purified an enzyme from sheep liver which catalyzes the reduction by DPNH of *S*-acetoacetyl-*N*-acetyl

thioethanolamine to the corresponding β-hydroxyacyl ester. It was during studies of this reductive process that Lynen first recognized the influence of pH on the equilibrium of the oxido-reduction.

Wakil *et al* (72) have measured the equilibrium of the reaction:



$$\beta\text{-Hydroxybutyryl CoA} + \text{DPN}^+ \rightleftharpoons \text{acetoacetyl CoA} + \text{DPNH} + \text{H}^+ \quad (39)$$

The value of K defined by the expression:

$$K = \frac{[\text{acetoacetyl CoA}][\text{DPNH}][\text{H}^+]}{[\beta\text{-hydroxyacyl CoA}][\text{DPN}^+]} \quad (40)$$

is 6.3×10^{-11} at pH 7.0 and 25°. The K value for the corresponding C_6 ester is 2.5×10^{-11} under the same experimental conditions The E_0' value (pH 7.0, 25°) for the β-hydroxybutyryl CoA-acetoacetyl CoA

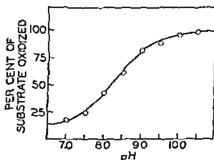


FIG 3 Per cent of β-hydroxybutyryl CoA oxidized (at equilibrium) in the β-hydroxyacyl dehydrogenase system as a function of pH (72)

system which can be calculated from the corresponding K value is −0.224 V Compare the potential of this system with the E_0' of the DPN^+—DPNH couple (−0.318 V)

Figure 3 shows how the per cent oxidation of β-hydroxybutyryl CoA by DPN increases over the pH range 7–11

In the presence of Mg^{++} the equilibrium is shifted in favor of β-ketoacyl CoA formation This has been shown by Stern (73) to be a consequence of complex formation between the metal ion and the β-ketoacyl CoA The proportion of enolate ion in the metal complex is much higher than that in the uncomplexed ester. The enolate ion is in fact responsible for the absorption band at 303 mμ shown by all β-ketoacyl CoA esters (64).

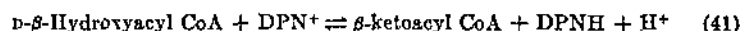
The beef liver enzyme of Wakil *et al* (72) and the sheep liver enzyme Lynen *et al* (64) are both specific for the L-isomers of the β-hydroxyacyl A esters—the same enantiomorphs which are formed by the action of

hydrase on *trans*-α,β-enoyl CoA esters It has long been known that liver also contains a pyridinoprotein which acts on β-hydroxybutyric acid as well as higher homologs (*74*) This enzyme (β-hydroxybutyric dehydrogenase) however, is specific for the D-isomers of these acids (*74*, *75*). It has no action on the D- or L-β-hydroxyacyl coenzyme A esters

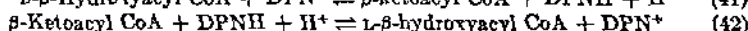
β-Hydroxyacyl dehydrogenase has recently been prepared in crystalline form by Stern (*76*) from pig heart Studies with this highly purified preparation by Marcus *et al* (*77*) have shown that the reversible transfer of hydrogen from reduced substrate to DPN, like analogous DPN-linked dehydrogenases studied previously (*78*, *79*), is direct and stereospecific In the present instance the hydrogen transfer is β-stereospecific for the nicotinamide ring of DPN

Wakil (*80*) has described still another pyridinoprotein from beef liver which appears to be specific for the D-isomers of β-hydroxyacyl CoA esters. This enzyme acts upon the products formed by the action of enoyl hydrase on the *cis*-α,β-enoyl CoA esters

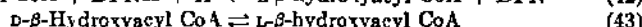
When the D- and L-acyl dehydrogenases are present together it is possible to catalyze the interconversion of D- and L-isomers as shown by the following equations



$$\text{D-}\beta\text{-Hydroxyacyl CoA} + DPN^{+} \rightleftharpoons \beta\text{-ketoacyl CoA} + DPNH + H^{+} \qquad (41)$$



$$\beta\text{-Ketoacyl CoA} + DPNH + H^{+} \rightleftharpoons \text{L-}\beta\text{-hydroxyacyl CoA} + DPN^{+} \qquad (42)$$



$$\text{D-}\beta\text{-Hydroxyacyl CoA} \rightleftharpoons \text{L-}\beta\text{-hydroxyacyl CoA} \qquad (43)$$

Stern *et al* (*69*) have also studied this interconversion but have attributed it to a racemase which does not require the presence of DPN and which does not depend upon the simultaneous operation of two separate pyridinoprotein enzymes sharing the same prosthetic group and forming the same product of dehydrogenation

Some recent studies of Von Korff (*81*) suggest a new wrinkle in the pathway for the metabolism of acetoacetate in heart muscle He finds that acetoacetate is rapidly reducible by DPNH even aerobically to β-hydroxybutyrate The D-isomer thus formed is then converted to its coenzyme A ester by an acyl thiokinase which in turn is oxidized to

way of the citric

Wakil probably

5 β-*Ketoacyl Thiolase*

Goldman (*82*) has isolated from beef liver mitochondria an enzyme which catalyzes the CoA-dependent cleavage of β-ketoacyl CoA esters from C_4 to at least C_8 Since the activity of the enzyme with β-ketooctanoyl CoA is higher than with β-ketobutyryl CoA as substrate it is

probable that the action of the enzyme covers the entire gamut of physiological chain length, i e., *from C_4 to C_{18}* However, Stern (*83*) has extensively purified a thiolase from pig heart muscle which is specific for acetoacetyl CoA

The Munich group have studied a similar enzyme prepared from sheep liver (*64*). They developed a simple assay for the enzyme which is based on the appearance or disappearance of the characteristic absorption band at 303 mμ referred to previously.

The cleavage of β-ketobutyryl CoA (AcAcSCoA) is reversible:



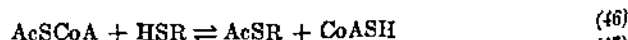
$$\text{AcAcSCoA} + \text{CoASH} \rightleftharpoons 2\ \text{AcSCoA} \tag{44}$$

The constant K defined by the expression:

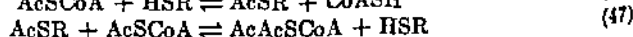
$$K = \frac{[\text{AcAcSCoA}][\text{CoASH}]}{[\text{AcSCoA}]^2} \tag{45}$$

has a value of $8\ 7 \times 10^{-5} \times \text{mole}^{-1}$ at pH 8.8, and of 6.0×10^{-6} at pH 8.5 (*31*) The equilibrium is thus strongly in favor of acetyl CoA formation

According to the Lynen school (*5, 64*) the prosthetic group of β-ketothiolase is an SH-group If the enzyme is represented as RSH then the interaction of the enzyme with AcSCoA leads to the formation of $RSAc^-$ (acetyl enzyme) and liberation of CoASH In turn $RSAc^-$ can interact with another molecule of AcSCoA to form AcAcSCoA and the original enzyme (RSH). According to this hypothesis there are two forms of active acetate, viz , AcSCoA and AcS enzyme

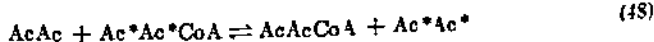


$$\text{AcSCoA} + \text{HSR} \rightleftharpoons \text{AcSR} + \text{CoASH} \tag{46}$$



$$\text{AcSR} + \text{AcSCoA} \rightleftharpoons \text{AcAcSCoA} + \text{HSR} \tag{47}$$

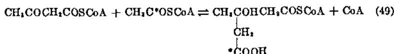
Beinert and Stansly independently arrived at a similar conclusion (*84*) These authors have shown that when carboxyl-labeled AcSCoA is exposed to an extract containing both thiolase and thiophorase the AcAcCoA thus formed is symmetrically labeled. When, however, unlabeled acetoacetate is present at the same time, the acetoacetate becomes labeled during the incubation with the mixture of enzymes and AcSCoA This label is asymmetrically distributed, i e , more in the carboxyl group than in the carbonyl. The higher the concentration of added acetoacetate the greater the asymmetry of label distribution Through the action of thiophorase unlabeled acetoacetyl CoA is formed from the added acetoacetate:

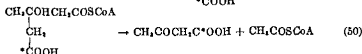


$$\text{AcAc} + \text{Ac}^*\text{Ac}^*\text{CoA} \rightleftharpoons \text{AcAcCoA} + \text{Ac}^*\text{Ac}^* \tag{48}$$

Since an opportunity is now provided for generating unlabeled acetyl enzyme (acetyl-thiolase) by reversal of Eq. (47), acetoacetyl CoA newly formed via thiolase would tend to be less intensively labeled in the carbonyl carbon

More recently Lynen *et al* (*13*) have proposed a new scheme to account for the observed asymmetry of labeling in acetoacetate. If the enzyme systems which form (*14*) and degrade (*16*) β-hydroxy-β-methyl glutaryl CoA (HMG CoA) are coupled together, the first two carbons of the starting acetoacetyl CoA are replaced by the acetyl moiety of acetyl CoA (*15*)·



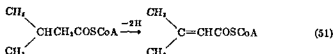


Thus unlabeled acetoacetyl CoA (such as the residuum left after oxidation of long-chain carboxyl-labeled fatty acids) in the presence of labeled acetyl CoA will lead to the formation of carboxyl-labeled acetoacetate.

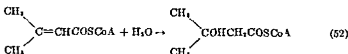
E Oxidation of Branched-Chain Fatty Acids

Coon and his colleagues have now elucidated at the enzyme level the sequence of reactions by which isovalerate (derived from leucine) (*16*, *85*) and α-methylbutyrate (derived from isoleucine) are oxidatively degraded (*86*)

Isovalerate in the form of its coenzyme A ester is dehydrogenated by acyl dehydrogenase to β-methylcrotonyl CoA



The product is hydrated to β-hydroxyisovaleryl CoA by enoyl hydrase:



At this stage there is a metabolic innovation. CO_2 is taken up with formation of β-hydroxy-β-methylglutaryl CoA:

$$(CH_3)_2COHCH_2COSCoA + CO_2 \xrightarrow{ATP} (CH_3)(COOHCH_2)COHCH_2COSCoA \quad (53)$$

This carboxylation reaction is mediated by ATP.

Lynen (*87*) has recently provided evidence for a different scheme. β-methylcrotonyl CoA is first carboxylated in the presence of ATP to form β-methylglutaconyl CoA, as shown in Eq. (54); the latter is then hydrated to form β-hydroxy-β-methylglutaryl CoA [Eq. (55)].

$$(CH_3)_2C{=}CHCOSCoA + CO_2 \xrightarrow{ATP} (CH_3)(COOHCH_2)C{=}CHCOSCoA \quad (54)$$

$$(CH_3)(COOHCH_2)C{=}CHCOSCoA + H_2O \longrightarrow (CH_3)(COOHCH_2)COHCH_2COSCoA \quad (55)$$

Finally β-hydroxy-β-methylglutaryl CoA is cleaved by a special enzyme (not β-ketoacylthiolase) into acetoacetate and acetyl CoA (*16*):

$$COOHCH_2C(CH_3)OHCH_2COSCoA \rightarrow COOHCH_2COCH_3 + AcSCoA \quad (56)$$

This cleavage enzyme requires the presence of Mg^{++} or Mn^{++} and some thiol compound such as cysteine, glutathione, or thioethanolamine

The degradation of α-methylbutyryl CoA to acetyl CoA and propionyl CoA proceeds as follows (*86*, *88*).

[illegible] (57)

[illegible] (58)

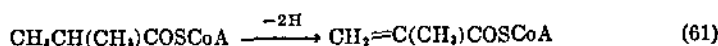
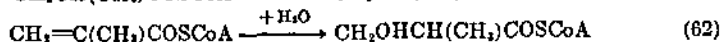
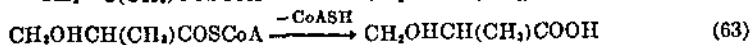
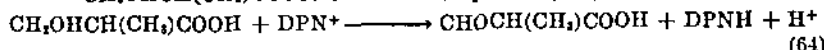
[illegible] $+ \ldots H^+$ (59)

$$CH_3COCH(CH_3)COSCoA + CoA \longrightarrow CH_3COSCoA + CH_3CH_2COSCoA \quad (60)$$

The enzymes which implement this sequence are respectively: acyl dehydrogenase, enoyl hydrase, β-hydroxyacyl dehydrogenase, and finally β-ketoacylthiolase.

The terminal oxidation of isobutyryl CoA (which is formed in the course of valine metabolism) has also been mapped out by Coon *et al.*

(*89, 90a*), Atchley (*90b*), and by Kinnory *et al* (*90c*) Isobutyryl CoA, after oxidation and hydration by acyl dehydrogenase and enoyl hydrase respectively, undergoes deacylation and reduction to form methyl malonyl semialdehyde

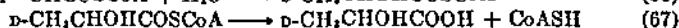

$$CH_3CH(CH_3)COSCoA \xrightarrow{-2H} CH_2{=}C(CH_3)COSCoA \quad (61)$$

$$CH_2{=}C(CH_3)COSCoA \xrightarrow{+H_2O} CH_2OHCH(CH_3)COSCoA \quad (62)$$

$$CH_2OHCH(CH_3)COSCoA \xrightarrow{-CoASH} CH_2OHCH(CH_3)COOH \quad (63)$$

$$CH_2OHCH(CH_3)COOH + DPN^+ \longrightarrow CHOCH(CH_3)COOH + DPNH + H^+ \quad (64)$$

It is possible that methyl malonyl semialdehyde may undergo either transamination to β-aminoisobutyrate or decarboxylation to propionate.

F Oxidation of Propionic Acid

Mahler and Huennekens (*91*) have presented indirect evidence derived from studies on mitochondrial preparations that propionate is converted to pyruvate by way of the sequence of reactions shown in Eqs (65–69) At the time their work was carried out the concept of acyl CoA's had not been developed, but their results pointed to an active form of propionate which we shall assume to be the CoA derivative


$$CH_3CH_2COSCoA \xrightarrow{-2H} CH_2{=}CHCOSCoA \quad (65)$$

$$CH_2{=}CHCOSCoA + H_2O \longrightarrow \text{D-}CH_3CHOHCOSCoA \quad (66)$$

$$\text{D-}CH_3CHOHCOSCoA \longrightarrow \text{D-}CH_3CHOHCOOH + CoASH \quad (67)$$

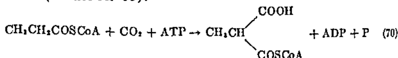
$$\text{D-}CH_3CHOHCOOH \longrightarrow \text{L-}CH_3CHOHCOOH \quad (68)$$

$$\text{L-}CH_3CHOHCOOH + DPN \longrightarrow CH_3COCOOH + DPNH \quad (69)$$

One of the postulated intermediates of the Mahler-Huennekens sequence (acrylyl CoA) has been isolated by Stadtman (*92*) from extracts of *Clostridium propionicum* as an intermediary in the conversion of propionate to β-alanyl CoA Another similar scheme has been suggested by Rendina and Coon (*90a*) which has been derived mainly by analogy from the pathway for isobutyryl CoA oxidation (see above) Propionyl CoA would thus pass through the following intermediates: acrylyl CoA, β-hydroxypropionyl CoA, free β-hydroxypropionate, and malonate semialdehyde. Malonate semialdehyde could then be converted to β-alanine by transamination In a system purified from *Clostridium kluyveri*, Vagelos (*92a*) has shown that β-hydroxypropionyl CoA is oxidized, in succession, to malonyl semialdehyde CoA and malonyl CoA by TPN.

The Lardy (*93–95*) and Ochoa (*96–98*) groups have accumulated

evidence at the enzyme level for the following pathway of propionic acid metabolism (cf also ref *99*).



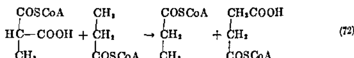
$$CH_3CH_2COSCoA + CO_2 + ATP \rightarrow CH_3CH(COOH)(COSCoA) + ADP + P \quad (70)$$

Finally, methyl malonyl CoA, the product of the carboxylation reaction, isomerizes to form succinyl CoA (*98, 100*).



$$CH_3CH(COOH)COSCoA \rightarrow COOHCH_2CH_2COSCoA \quad (71)$$

The first step, Eq. (70), has suggested the possibility of an active CO_2, but there is at present no evidence for a free intermediate. The carboxylation enzyme has been purified extensively without any indication that it may be resolved into several fractions. The isomerization of methyl malonyl CoA to succinyl CoA, Eq. (71), may proceed in such a fashion that propionyl CoA is actually cast in the role of a cofactor (*100*):



$$COSCoA{-}CH(COOH){-}CH_3 + CH_3CH_2COSCoA \rightarrow COSCoA{-}CH_2{-}CH_3 + CH_2(COOH){-}CH_2{-}COSCoA \quad (72)$$

G Fatty Acid Oxidation in Mitochondria

It may be of interest to reexamine some of the salient features of the process of fatty acid oxidation at the mitochondrial level in the light of the information which is now available from studies of the separated, soluble enzymes The following are three of the most characteristic features of mitochondrial fatty acid oxidation (*101*): (1) some member of the citric acid cycle must be undergoing oxidation as a prior condition for fatty acid oxidation, (2) ATP cannot replace the citric cycle metabolite or "sparker"; (3) 2,4-dinitrophenol completely paralyzes fatty acid oxidation

The sparking phenomenon is referable in part to the generation of ATP by oxidative phosphorylation. During the oxidation of any member of the citric acid cycle inorganic phosphate becomes esterified, and ATP is formed, which then drives the synthesis of the fatty acyl coenzyme A ester ATP cannot replace the sparker since the sparker has another function besides that of generating ATP, viz , that of generating by way of the citric cycle the condensing partner (oxalacetate) for acetyl CoA Unless acetyl CoA arising during β-oxidation can be condensed with oxalacetate to form citrate, the available CoA which is present in limiting

or catalytic amount is tied up as the thiol ester. Only as much acetyl CoA can be formed as there is total CoA. Thus the sparker has the dual role of facilitating a turnover of CoA through the condensation reaction and of generating ATP by oxidative phosphorylation. The inhibitory effect of 2,4-dinitrophenol is a consequence of the abolition of oxidative phosphorylation by this reagent (*102*).

In liver, ATP can replace the sparker completely by virtue of a new possibility opened by acetoacetyl CoA deacylase. Acetyl CoA formed by β-oxidation condenses with another molecule to form acetoacetyl CoA according to the usual β-ketothiolase reaction. Then acetoacetyl CoA is deacylated to free acetoacetate and CoA. In this way CoA can be regenerated without the citric acid cycle being involved. This special feature of liver explains the early observation of Lehninger (*103*) that ATP can replace the sparker in the oxidation of fatty acids under conditions where acetoacetate accumulates.

It may be appropriate to consider at this point the association of the enzymes which implement the fatty acid oxidation cycle with the mitochondrion—an association which was first established by Lehninger and [illegible]

cycle as well as with the mitochondrial electron transport system. ETF (the electron transfer flavoprotein) is one of the two links between the fatty acid cycle and the electron transport system while DPNH is the other. ETF transfers electrons derived from the fatty acyl coenzyme A esters to the heme chain of the electron transport system while DPNH formed during the oxidation of the β-hydroxyacyl CoA esters is oxidized by molecular oxygen by way of the terminal electron transfer system (*105*).

The bonds which hold the enzymes of the fatty acid oxidation cycle to one another and to the electron transfer system are usually ruptured by simple mechanical means such as prolonged homogenization in a blendor at high speeds. From all indications the mitochondrion-bound enzymes of the fatty acid oxidation cycle are not separate and freely diffusible. They constitute rather a tight complex and in this form they fit into the structural pattern of the mitochondrion.

H. FATTY ACID DEHYDROGENASE (UNSATURASE)

Various animal tissues (liver, skeletal muscle, heart, and testis) have been shown by several investigators—Mazza and Stolfi (*106*), Lang (*107*), Shapiro and Wertheimer (*108*), and Franke and Frehse (*109*)—to contain an enzyme which catalyzes the dehydrogenation of fatty acids with the

formation of an unsaturated fatty acid Thus stearic acid is dehydrogenated to oleic acid ($\Delta^{9,10}$-octadecenoic acid); palmitic acid is dehydrogenated to palmitoleic acid ($\Delta^{9,10}$-hexadecenoic acid), other acids, odd or even, of shorter chain length are dehydrogenated although progressively more slowly to corresponding unsaturated acids

Bloomfield and Bloch (*110*) have prepared an extract of yeast which catalyzes the dehydrogenation of palmitic acid This process requires the presence of both molecular oxygen and TPNH, and it thus resembles the hydroxylation reactions involved in the oxidation of phenylalanine to tyrosine and in the hydroxylation of steroids (*111, 112*).

I Fatty Acid Peroxidase

An alternate route of fatty acid oxidation not involving acyl esters of CoA has been studied by Stumpf and co-workers (*113–115*) in plant tissue Extracts of cotyledons of germinating peanut seedlings oxidize stearic, palmitic, and myristic acids with the loss of the carboxyl carbon as CO_2 (*113*) The reaction is stimulated by glycolic acid. More recent evidence indicates that these events are best described as a peroxidation of long-chain fatty acids by hydrogen peroxide which is generated by glycolic acid and glycolic oxidase (*115*) Evolution of CO_2 from the terminal carboxyl group is accompanied by the accumulation of a long-chain fatty aldehyde

II. Fatty Acid Synthesis

A Introduction

Whereas the general principles and even many of the details of fatty acid oxidation have been thoroughly clarified at the enzymic level, the sequence of events in fatty acid synthesis has been vaguely defined up to the present. Certainly there is compelling evidence that long-chain fatty acids such as palmitic acid arise by successive condensation of acetate units, but the manner in which this is effected is still in the process of being unraveled

Before considering the available information about cell-free systems of enzymes which catalyze fatty acid synthesis it may be profitable to review some pertinent observations made on the level of the whole animal which set a framework for any comprehensive picture of fatty acid synthesis.

The fact that dietary carbohydrate and protein give rise to fat has been known for almost a century. But it was not until 1926 that Smedley-MacLean and Hoffert (*116*) observed that yeast cells could grow and accumulate lipid when provided with acetate as the only source of carbon In recent years the origin of acetate from carbohydrate [by way of

pyruvate (*117–120*)] and from protein [by way of ketogenic amino acids such as leucine (*16*, *121*) and isoleucine (*86*, *122*)] has been conclusively demonstrated Consequently acetate, in the form of acetylCoA (*4*, *123*) was destined to lie at the intersection of the great trunk lines of glycolysis, the tricarboxylic acid cycle, and lipid metabolism

With the advent of isotopic tracer techniques introduced and developed by the Schoenheimer school (*124*) the concept of the idle, immobile fat storage depot had to be abandoned All the newly acquired isotopic evidence pointed to the lipids being in a state of dynamic balance between synthesis and degradation—both of which processes were going on simultaneously and continuously For example, it was observed that palmitic acid was being converted to stearic acid at the same time that stearic acid was being converted to palmitic acid (*125*, *126*) Tracers permitted the demonstration that in the formation of long-chain fatty acids from acetate in animal tissues every carbon atom in the chain must have originated from the carbon of acetate (*127–129*) Furthermore, the carboxyl end of one acetate unit must have condensed with the methyl end of the next acetate unit (*130*)

In view of this striking parallelism between fatty acid oxidation and synthesis the idea that the two processes are one and the same, except that they operate in opposite directions, seemed unavoidable In 1949 Stadtman and Barker (*131*) reported a series of brilliant experiments on the anaerobic synthesis of butyrate and caproate from ethyl alcohol in cell-free extracts of *Clostridium kluyveri* Since the same extract could carry out either the anaerobic synthesis of fatty acids from ethanol (by way of acetyl phosphate) or the oxidation of the same fatty acids to acetate (in the form of acetyl phosphate) the case for assuming the identity of the oxidative and synthetic process seemed overwhelming

The first rumble that this approach was too simple came from experiments carried out with the purified enzymes of the fatty acid oxidation cycle While acetyl CoA could be converted to butyryl CoA with a combination of these enzymes no evidence could be obtained for the synthesis of a fatty acid with more than four carbon atoms (*132*) This negative experiment could, of course, mean that some additional component essential for synthesis was lacking On the other hand, it could be a token that fatty acid synthesis at the enzymic level is not the reversal of fatty acid oxidation [see also ref (*133*)]

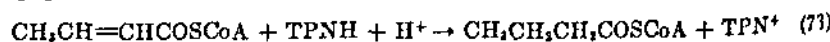
B Fatty Acid Synthesis in Cell-Free Systems of Animal Tissues

There have been essentially two basic approaches to the study of fatty acid biosynthesis in soluble systems One approach has been to

assume that in the economy of nature the enzymes of fatty acid oxidation, at least in part, are operative in synthesis, and that the intermediates encountered in the oxidation pathway also obtain in synthesis. The other stratagem has been to make no assumptions and to seek out systems and conditions which efficiently transform acetate into fatty acids.

Gurin's laboratory was the first to report fatty acid synthesis in a cell-free system (*134, 135*). Originally the system consisted of a homogenate of pigeon liver, but later a soluble system was described that required both the high-speed (100,000 *g*) centrifugal supernatant and *mitochondrial* extracts. It was found that citrate markedly stimulated fatty acid synthesis (*134*) and that the activity of the charcoal-treated system could be partially restored when supplemented with ATP, CoA, and DPN (*135*). A short time later Popják and Tietz described a similar system from mammary gland (*136, 137*), in which a clear-cut requirement for ATP, CoA, and DPN could be demonstrated. When a more purified preparation was tested Hele and Popják (*138*) and Hele *et al.* (*139*) found that DPNH oxidation closely paralleled the formation of short-chain fatty acids.

Langdon (*140, 141*) while studying fatty acid synthesis in rat liver homogenates (which had been freed of particles by high speed centrifugation), recognized yet another cofactor, viz., TPN. He made a further discovery that seemed to answer why TPN was necessary. The liver preparation catalyzed the reduction of crotonyl CoA by TPNH to butyryl CoA.



$$CH_3CH{=}CHCOSCoA + TPNH + H^+ \rightarrow CH_3CH_2CH_2COSCoA + TPN^+ \quad (71)$$

Recently Seubert *et al.* (*142*) have succeeded in isolating the reductase from liver mitochondria. This reductase in concert with highly purified thiolase, β-hydroxyacyl dehydrogenase, and enoyl hydrase (along with ancillary systems for generating DPNH and TPNH) was shown to effect a condensation of acetyl CoA with hexanoyl CoA leading to the formation of both octanoyl CoA and progressively smaller quantities of the longer chain acyl derivatives of CoA. The success of this demonstration depended on adding both DPNH and TPNH and all of the oxidation enzymes listed above. Also when hexanoyl CoA was omitted from the system there was no significant incorporation of acetyl CoA into fatty acids. This experiment substantiated the contention that mitochondria do possess a system which can at least increase the chain length of fatty acids by a process that is essentially a reversal of fatty acid oxidation [also cf. ref. (*143, 144*)].

Recent studies by Gibson, Wakil, and co-workers (*145–147*) have demonstrated the existence of a new system for fatty acid biosynthesis

which does not require the participation of the fatty acid oxidation enzymes Two purified fractions from the nonparticulate supernatant of pigeon liver catalyze the synthesis of palmitate from acetyl CoA provided that four obligatory cofactors are added, viz ATP, HCO_3^-, Mn^{++}, and TPNH (see Table IV) [4]

Although acetyl CoA is the starting point for synthesis it is essential to add ATP—an observation which suggested a new activating function for ATP Another development which clearly distinguishes this system from the mitochondrial system of Seubert *et al* (*142*) is the finding that bicarbonate is uniquely required for palmitate synthesis (*145, 149*).

TABLE IV
COMPONENTS OF THE FATTY ACID SYNTHESIZING SYSTEM OF PIGEON LIVER

	Acetyl CoA (μmoles) incorporated into fatty acid
Complete system[a]	0 394
No ATP	0 000
No TPNH	0 000
No Mn^{++}	0 030
No HCO_3^-	0 010
No R_{1g} (or R_{2g})	0 000

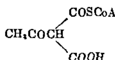
[a] Complete system 50 μmoles histidine buffer (pH 6 5), 2 μmoles ATP, 0 6 μmoles $MnCl_2$, 0 6 μmoles acetyl CoA, 0 8 μmoles TPNH, 8 μmoles $KHCO_3$, 1 mg of R_{1g}, and 0 8 mg R_{2g} Total volume was 1 0 ml, and each sample was incubated 30 minutes at 38°

Yet, no $HC^{14}O_3^-$ is incorporated into palmitate Wakil (*150*) subsequently isolated a malonyl-containing intermediate (later identified as malonyl CoA) after incubating acetyl CoA with ATP, Mn^{++}, HCO_3^- and one of the two purified liver fractions (designated R_{1g}) Moreover the malonyl intermediate is quantitatively converted to palmitate by the second enzyme fraction (R_{2g}) and TPNH Thus the two protein fractions serve entirely different functions R_{1g} catalyzes the carboxylation of acetyl CoA to malonyl CoA in presence of ATP and Mn^{++} while R_{2g} catalyzes the reductive condensation of malonyl CoA units to long-chain fatty acids with TPNH as reducing agent

The problem of how malonyl CoA undergoes reductive condensation was simplified by the discovery of Wakil and Ganguly (*151*) that acetyl CoA is incorporated into palmitate in presence of R_{2g} and TPNH *only* when malonyl CoA is present. Furthermore the rate of fatty acid synthesis from malonyl CoA is greatly enhanced by the addition of acetyl CoA These twin observations suggested a condensation between acetyl

[4] Stumpf *et al* have confirmed these findings in a system derived from avocado (*148*)

CoA and malonyl CoA with formation of a product with 5 carbon atoms, perhaps of the composition shown below:



This can be looked upon as a carboxy derivative of acetoacetyl CoA Since one of the two carboxyl groups is lost during synthesis it must be presumed that acetyl malonyl CoA is reductively decarboxylated to butyryl CoA Indeed it has been demonstrated by Wakil and Ganguly (*151*) that malonyl CoA can condense not only with acetyl CoA but equally well with butyryl CoA, hexanoyl CoA, octanoyl CoA, etc. and that each of these condensations leads to the synthesis of palmitate. It is only the acyl CoA esters which condense β-Hydroxy and β-ketoacyl CoA esters or α-β unsaturated acyl CoA esters do not replace the fatty acyl CoA esters in these condensations

R_{2a} contains an enzyme which catalyzes the decarboxylation of malonyl CoA to acetyl CoA and CO_2 As a consequence of this enzymic process malonyl CoA can give rise to long-chain fatty acids Condensing partner (acetyl CoA) is generated as soon as malonyl CoA is exposed to R_{2a} Very likely the same decarboxylating enzyme acts upon the higher homologs ethyl malonyl CoA, butyryl malonyl CoA, etc to liberate the corresponding longer chain acyl CoA esters The intermediates in the reductive decarboxylation of acyl malonyl CoA esters to acyl CoA esters have yet to be identified.

Brady *et al* (*152*) have discussed the possibility of an aldol condensation of the Knoevenagel type between acetaldehyde and malonyl CoA in fatty acid biosynthesis by analogy with a step in the synthesis of sphingosine. However, acetaldehyde or other aldehydes cannot replace acetyl CoA in the Gibson-Wakil system and so this interesting possibility suggested by Brady probably does not apply.

The Gibson-Wakil system, in contrast to the Seubert system (*142*), catalyzes the synthesis of long-chain fatty acids *de novo* from acetyl CoA As was demonstrated previously in earlier preparations from avian liver (*153*), the major synthetic product of the purified system is free palmitic acid with much smaller quantities of myristic and stearic acid (see Fig. 4) No short-chain fatty acids accumulate, nor do added short-chain fatty acids facilitate acetyl CoA incorporation

The enzyme fractions of the Gibson-Wakil system are prepared from the particle-free supernatant of avian liver (*145*) The active enzymes in two crude ammonium sulfate fractions (R_1, 0–30% saturation and R_2,

30–40% saturation) are separately adsorbed on and then selectively eluted from calcium phosphate gel. The gel eluate of R_1 is further purified by high-speed centrifugation (*146*). This latter fraction (R_{1g}) which

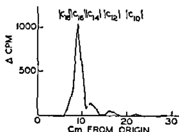


FIG 4 Distribution of synthesized long-chain fatty acids as determined by chromatographic separation

catalyzes the carboxylation of acetyl CoA to malonyl CoA, is exceptionally rich in protein-bound biotin Figure 5 illustrates the close correlation between the specific enzyme activity of R_{1g} and its biotin content Avidin—the egg white protein that specifically binds biotin—inhibits fatty acid synthesis This inhibitory effect is prevented by preincubating the system with free *d*-biotin (*154*)

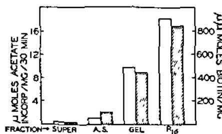


FIG 5 Comparison of specific enzyme activity and biotin content of fractions obtained during the purification of R_{1g}

The fact that the carboxylation of acetyl CoA by R_{1g} involves both biotin and ATP suggested the possibility that ATP energizes the carboxylation of biotin and that the biotin-CO_2 complex is the actual carboxylating agent Indeed it can be shown that after interaction of R_{1g} with P^{32}-labeled ATP label is incorporated into some group of R_{1g} Lynen has reported experiments which point to an ADP derivative of biotin as an intermediate in the formation of biotin-CO_2. Presumably CO_2 displaces the ADP group which is attached to one of the N atoms in the biotin molecule (*155*).

The purified fractions R_{1a} and R_{2a} are the only enzyme components required for synthesis (see Table IV). Yet they do not contain any appreciable concentration of the enzymes of the fatty acid oxidation sequence. Nor has the enzyme that catalyzes the reduction of α,β-unsaturated acyl CoA by TPNH [see Eq (73)] been detected in the most purified system (*145*).

Thus for the present it must be assumed that at least two systems are operative in the biosynthesis of fatty acids One, which is derived from mitochondria, is chiefly concerned with the lengthening of preexisting short-chain fatty acids via a reversal of the β-oxidation cycle. The second system, localized principally in the nonparticulate cytoplasm of the cell, catalyzes the synthesis of palmitate *de novo* from acetyl CoA without the participation of the classic fatty acid oxidation enzymes

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Ascorbic Acid

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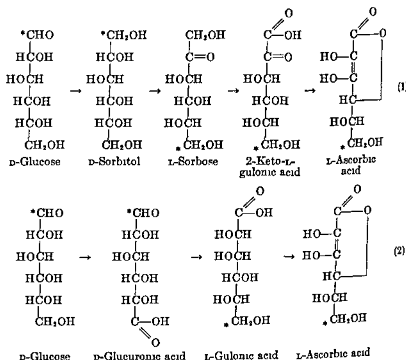
I. Introduction

The importance of L-ascorbic acid in the prevention of scurvy has been known for many years but definitive information on its mode of action at the molecular level is not available L-Ascorbic acid is synthesized in a great variety of plants and in all known mammals except for primates and the guinea pig In recent years mechanisms for the biosynthesis of L-ascorbic acid in plants and animals, along with those involved in its catabolism, have been presented These studies have disclosed new enzymic reactions for such compounds as D-glucuronic acid, D-galacturonic acid, L-gulonic acid, and L-xylulose As a consequence of this work a new route of glucose metabolism, the glucuronic acid pathway, has been demonstrated in mammalian tissues This chapter is concerned primarily with the metabolic pathways involved in the formation and degradation of L-ascorbic acid in animals and plants Comprehensive reviews on the interaction of L-ascorbic acid with various enzymes, as well as information on the occurrence, chemistry, and physiological functions of the vitamin are available (*1–4*)

II. Biosynthesis of L-Ascorbic Acid in Animals

corporation of C^{14} into urinary L-ascorbic acid was determined (Table I) Glucose-1-C^{14} was converted to L-ascorbic acid labeled chiefly in C-6 and glucose-6-C^{14} was converted to L-ascorbic acid labeled chiefly in C-1. These results indicate that the carbon chain of glucose is converted intact to L-ascorbic acid by a mechanism in which it undergoes an inversion of configuration In these studies the synthesis of L-ascorbic acid was stimulated by the administration of Chloretone, but a similar precursor role of glucose for L-ascorbic acid has been observed in rats not receiving the drug (*8*).

Two possible pathways, reactions (1) and (2), have been considered for the biosynthesis of L-ascorbic acid which would account for an inversion of the carbon chain of glucose.



According to reactions (1) and (2), C-1 of glucose would become C-6 of L-ascorbic acid as indicated by the asterisks

Reaction (1) This pathway was ruled out by results of experiments (*9*) employing L-sorbose-6-C^{14} If L-sorbose is an intermediate it would be expected that L-sorbose-6-C^{14} would yield C-6 labeled L-ascorbic acid However, this was found not to be the case since administration of

L-sorbose-6-C^{14} to rats resulted in L-ascorbic acid labeled about equally in C-1 and C-6 In fact, any C^{14} incorporated into L-ascorbic acid following administration of the labeled L-sorbose may be accounted for by the intermediate formation of glucose In addition, experiments employing uniformly C^{14}-labeled 2-keto-L-gulonic acid showed no detectable conversion of this compound to L-ascorbic acid in the rat (*10*)

Reaction (*2*) Considerable evidence is now available for the conversion of glucose to L-ascorbic acid by this pathway in animals Isherwood and co-workers (*11*) reported that the administration of D-glucuronolactone and L-gulonolactone to rats produced an increase in the urinary excretion of L-ascorbic acid Definitive evidence for the conversion of the intact carbon chain of D-glucuronolactone and L-gulonolactone to L-ascorbic acid in rats came from isotopic experiments (Table I) Administration of uniformly labeled D-glucuronolactone to Chloretone-treated rats resulted in the excretion of uniformly labeled L-ascorbic acid (*12*) Carboxyl-labeled D-glucuronolactone and L-gulonolactone yielded carboxyl-labeled L-ascorbic acid in both normal and Chloretone-treated rats (*13*). In addition, the conversion of D-glucose-1-C^{14} to urinary D-glucuronic acid and L-gulonic acid has been shown in rats receiving barbital and Chloretone, drugs which are known to stimulate the synthesis of L-ascorbic acid (*14, 15*).

Further evidence for reaction (*2*) has come from the finding that enzymes exist in liver which convert D-glucuronic acid and L-gulonic acid to L-ascorbic acid (*16, 17*) A TPN-linked dehydrogenase present in the soluble fraction reversibly catalyzes the reduction of D-glucuronic acid to L-gulonic acid (*16, 18, 19*). A lactonase has also been demonstrated in the soluble fraction which carried out the interconversion of L-gulonic acid with L-gulonolactone (*20*) In addition, enzymes are present in liver microsomes which convert L-gulonolactone to L-ascorbic acid (*16, 21*) This microsomal system has been solubilized and evidence was obtained for 2-keto-L-gulonolactone as the most likely intermediate in this reaction (*22*)

The only known mechanism for the conversion of glucose to D-glucuronic acid is via the oxidation of uridinediphosphoglucose to uridinediphosphoglucuronic acid, a reaction catalyzed by a DPN-linked enzyme in the soluble fraction of liver (*23, 24*). An enzyme has been demonstrated in liver microsomes which forms glucuronides by transferring D-glucuronic acid from UDPGA to a receptor (*23, 24*). Although the mechanisms involved in the formation of glucuronides through uridine nucleotides have been known for several years, the relevance of these reactions to the biosynthesis of L-ascorbic acid has been indicated only recently by Evans *et al* (*25, 25a*) In this study, galactose-1-C^{14} was found

to be a considerably better precursor of L-ascorbic acid in rats than is glucose-1-C^{14} (Table I) The L-ascorbic acid formed from galactose-1-C^{14} had about 92% of its total activity in C-6 compared to 60% for that from glucose-1-C^{14} indicating that galactose is converted to L-ascorbic acid without the intermediate formation of glucose. In addition, rat liver was shown to have the enzymes required for the conversion of D-galactose to L-ascorbic acid through D-glucuronic acid It is of particular importance that an enzyme system has been found in rat liver microsomes which converts UDP glucuronic acid to glucuronic acid-1-PO_4 (*25*) Earlier studies by Ginsburg *et al.* (*26*) have pointed out a similar system in the particulate fraction of rat kidney, capable of converting UDP glucuronic acid to D-glucuronic acid, presumably via D-glucuronic acid-1-PO_4 However, this kidney system is not required for L-ascorbic acid formation since D-glucose-1-C^{14} is converted to labeled D-glucuronic acid and L-ascorbic acid in nephrectomized rats to about the same extent as in normal rats (*25*) Based upon currently available evidence, the most likely over-all scheme for the biosynthesis of L-ascorbic acid from glucose or galactose is reaction (*3*).

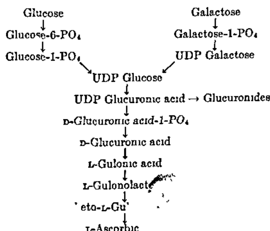


TABLE I
CONVERSION OF VARIOUS LABELED COMPOUNDS TO L-ASCORBIC ACID IN RATS

Compound	% Conversion to L-ascorbic acid	% of total L-ascorbic acid C^{14} in		Ref.
		C-1	C-6	
D-Glucose-U-C^{14}	0 40			*(5)*
D-Glucose-1-C^{14}	0 55	11	56	*(6)*
D-Glucose-6-C^{14}	0 50	61	15	*(7)*
L-Sorbose-U-C^{14}	0 40			*(9)*
L-Sorbose-6-C^{14}	0 30	27	27	*(9)*
D-Glucuronolactone-U-C^{14}	2 2			*(12)*
D-Glucuronolactone-6-C^{14}	1 2	90		*(13)*
L-Gulonolactone-1-C^{14}	3 6	85		*(13)*
D-Galactose-1-C^{14}	0 43[a]		92	*(25)*
D-Glucose-1-C^{14}	0 045[a]	10	60	*(8, 25, 25a)*

[a] In these experiments the % conversion was estimated from the amount of C^{14} in body L-ascorbic acid at 24 hours by a technique based on the unusually long half life of L-ascorbic acid in the rat *(13)* In the other experiments the % conversion was estimated from the amount of C^{14} excreted as urinary L ascorbic acid during a 24-hour period after administration of the labeled compounds to Chloretone-treated rats *(5)*

unable to convert L-gulonolactone to L-ascorbic acid For instance, it has been demonstrated *in vivo* that guinea pigs could not convert D-glucuronolactone-6-C^{14} and L-gulonolactone-1-C^{14} to L-ascorbic acid, in contrast, rats converted appreciable amounts of both compounds *(13)*. Results which appeared almost simultaneously from two laboratories further pointed out the nature of this missing step Lehninger and co-workers *(16, 27)* found no net synthesis of L-ascorbic acid from L-gulonolactone in human, monkey, and guinea pig liver homogenates as measured colorimetrically *(28)* Under these conditions there was an appreciable synthesis of the vitamin from L-gulonolactone in rat, mouse, rabbit, and dog liver homogenates Burns and co-workers *(21, 29)* reported no detectable conversion of L-gulonolactone-1-C^{14} to labeled L-ascorbic acid in human, monkey, and guinea pig liver homogenates, conversion being less than one one-hundredth of that obtained in the rat (Table II).

Man, monkey, and guinea pig can carry out the various steps required in reaction (3) for the biosynthesis of the vitamin except the conversion of L-gulonolactone to L-ascorbic acid. The actual missing step in these species is most likely the initial oxidation of L-gulonolactone to 2-keto-L-gulonolactone. This keto derivative is a tautomeric form of L-ascorbic acid and presumably would be transformed spontaneously into the vitamin

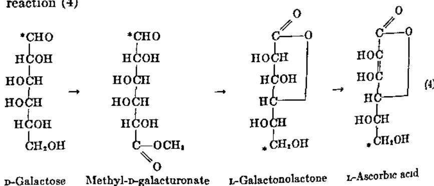
TABLE II
CONVERSION OF L-GULONOLACTONE-1-C^{14} TO L-ASCORBIC ACID IN RAT, GUINEA PIG, MONKEY, AND HUMAN LIVER[a]

Species	% Conversion	
	Homogenate	Microsomes
Rat	8 0	10 0
Guinea pig	<0 05	<0 05
Monkey	<0 07	—
Man	<0 07	—

[a] In these experiments 5 0 mg of L-gulonolactone was incubated for 90 min at 37° under air in 5 ml of 10% homogenate (or microsomes from an equivalent amount of liver) in 0 15 M phosphate buffer (pH 7 20) containing 0 13 M sucrose L-Ascorbic acid was isolated after addition of carrier (150 mg) from a 5% trichloroacetic acid extract of the incubation mixture as its 2,4-dinitrophenylosazone derivative

IV. Biosynthesis of L-Ascorbic Acid in Plants

Isherwood and co-workers (*11, 30, 31*) demonstrated that L-gulonolactone, L-galactonolactone, D-glucuronolactone, and methyl-D-galacturonate were converted to L-ascorbic acid in cress seedlings From their studies, they proposed two pathways for the synthesis of L-ascorbic acid in plants, reaction (2), the same as they postulated for animals, and reaction (4)



Recent studies by Loewus *et al* (*32–34*) in the detached ripening strawberry and in the germinating cress seedling do not support these mechanisms as major pathways for the biosynthesis of the vitamin in plants since no evidence for an inversion between D-glucose and L-ascorbic acid was found For instance, D-glucose-1-C^{14} yielded L-ascorbic acid containing 65–70% of its activity in C-1 and 14–19% in C-6. With D-glucose-6-C^{14}, 73% of the total activity in L-ascorbic acid was present in C-6 and 24% in C-1. When D-galactose-1-C^{14} was used as the sub-

strate the L-ascorbic acid had about the same labeling in C-1 and C-6. These findings are in marked contrast to the data obtained in rats which showed that both C-1 of glucose and C-1 of galactose became C-6 of L-ascorbic acid (Table I). Thus, different pathways must exist in plants and animals for the biosynthesis of L-ascorbic acid. Loewus and co-workers have postulated the scheme in Fig. 1 as a possible mechanism for the conversion of glucose to L-ascorbic acid which would be in accord

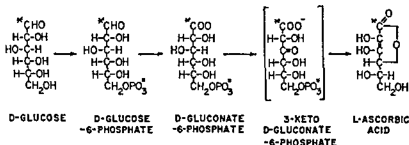


FIG. 1. Postulated scheme for the biosynthesis of L-ascorbic acid in plants. The asterisks denote the fate of C-1 of glucose.

with their isotopic data in plants. According to this scheme C-1 of glucose would become C-1 of L-ascorbic acid as indicated by the asterisks. 3-Keto-D-gluconate-6-PO_4 has been postulated to be the intermediate involved in the formation of D-ribulose-5-PO_4 according to the reactions of the hexose monophosphate shunt (*35*). For the conversion of this keto acid to L-ascorbic acid, an epimerization reaction at C-5 would be required followed by the loss of phosphate from the resulting compound and its subsequent lactonization and enolization. Conclusion on the importance of the mechanism in Fig. 1 in the biosynthesis of L-ascorbic acid must await information on the enzymic steps involved.

V. Catabolism of L-Ascorbic Acid in Animals

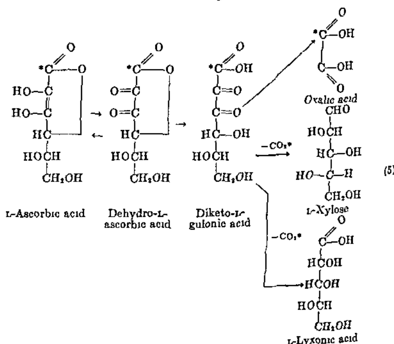
Studies with L-ascorbic acid, labeled in various positions with C^{14}, have shown that the vitamin is extensively oxidized to respiratory CO_2 in rats and guinea pigs (Table III). In contrast no conversion of L-ascorbic acid-1-C^{14} to respiratory CO_2 was detected in man. L-Ascorbic acid disappears slowly in man with a half-life of about 16 days (*41*) compared to a half-life of about 3 days for the guinea pig (*37, 42*). This is correlated with the much longer time it takes human subjects to show signs of scurvy. It requires about 4 months for man on a vitamin C free diet to develop scurvy, while the guinea pig is scorbutic in about 3 weeks.

TABLE III
C^{14} IN RESPIRATORY CO_2 AND URINE AFTER ADMINISTRATION OF LABELED L-ASCORBIC ACID TO VARIOUS ANIMAL SPECIES

Compound*	Animal	Collection period (days)	Per cent of administered C^{14} found in CO_2	Per cent of administered C^{14} found in Urine	Ref
L-Ascorbic acid-1-C^{14}	Guinea pig	1	20	7 2	(36)
L-Ascorbic acid-1-C^{14}	Guinea pig	10	70	20	(36)
L-Ascorbic acid-U-C^{14}	Guinea pig	1	22	11	(37)
L-Ascorbic acid-6-C^{14}	Guinea pig	1	18	6 0	(38)
L-Ascorbic acid-2,3,4,5,6-C^{14}	Guinea pig	1	28	7 5	(39)
L-Ascorbic acid-1-C^{14}	Rat	1	14	25	(40)
L-Ascorbic acid-1-C^{14}	Man	10	<5 0	42	(41)

* The labeled compounds were administered intraperitoneally to guinea pigs and rats and intravenously to man

On the basis of available evidence, the most likely scheme for the catabolism of the vitamin in animals is given in reaction (5).



The formation of dehydroascorbic acid and 2,3-diketogulonic acid from L-ascorbic acid was postulated initially by Penny and Zilva (43), and

evidence for these intermediates has come from numerous studies. The conversion of L-ascorbic acid to dehydroascorbic acid is reversible but the formation of 2,3-diketogulonic acid occurs irreversibly.

L-Ascorbic acid is converted to urinary oxalate in man (41), guinea pig (36), and rat (40) presumably through the intermediate formation of dehydroascorbic acid and diketogulonic acid However, the mechanism by which oxalate is formed is unknown since attempts to demonstrate

TABLE IV
INCORPORATION OF ISOTOPE INTO GLUCOSE FOLLOWING ADMINISTRATION OF VARIOUS LABELED COMPOUNDS

Compound	Per cent of administered isotope in glucose	Per cent of total glucose isotope in			Ref
		C-1	C-3	C-6	
	Animal[a]				
L-Ascorbic acid-6-C^{14}	0 28	38	6 5	20	(38)
Dehydroascorbic acid-6-C^{14}	1 4	49	6 5	27	(38)
Diketogulonic acid-6-C^{14}	2 1	41	5 6	37	(38)
L-Ascorbic acid-3,4,5,6-C^{14}		15		7	(44)
L-Ascorbic acid-2,3,4,5,6-C^{14}	0 52				(39)
L-Gulonolactone-6-C^{14}	22	55	17	3 0	(49)
L-Sorbose-6-C^{14}	12	44		34	(9)
	Plant[b]				
L-Ascorbic acid-6-C^{14}	—	51	5	34	(34)

[a] The conversion of labeled compounds to liver glycogen was measured either in guinea pigs or rats.
[b] The conversion of L-ascorbic acid-6-C^{14} to sucrose-derived glucose was measured in a ripening strawberry

significant conversion of L-ascorbic acid to oxalate in rat and guinea pig tissues have not been successful (44, 45) It should be noted that the conversion of L-ascorbic acid to oxalate and L-threonic acid can be readily demonstrated under nonbiological conditions (46, 47).

Chan *et al* (44) have shown that dehydroascorbic acid is decarboxylated in guinea pig liver homogenates with the formation of L-xylose as a product In addition, a system has been described in rat kidney which decarboxylates L-ascorbic acid through the intermediate formation of dehydroascorbic acid and diketogulonic acid (45). L-Lyxonic and L-xylonic acids were identified as products of this reaction in rat kidney (48)

In order to obtain further information on the pathways of metabolism of the vitamin in animals, the conversion of labeled L-ascorbic acid, dehydroascorbic acid and diketogulonic acid to liver glycogen was measured (Table IV) Carbon-6 labeled L-ascorbic acid, dehydroascorbic acid, and diketogulonic acid were converted to glycogen which contained the

major fraction of the total C^{14} in its glucose residues in C-1 and C-6. This isotopic pattern is markedly different from that obtained in similar experiments with L-gulonolactone-6-C^{14}. According to the data in Table IV, L-gulonolactone-6-C^{14} yields glycogen which is labeled predominantly in C-1 and C-3. These findings argue against any appreciable conversion of L-ascorbic acid to glycogen via L-xylulose as postulated for its precursor, L-gulonolactone (*49*). The appearance of isotope predominantly in C-1 and C-6 of glucose derived from C-6 labeled L-ascorbic acid, dehydroascorbic acid and diketogulonic acid suggests the intermediate formation of trioses Since these precursors are of the L-configuration, one triose from each should be of that configuration. The ability of L-trioses to participate in glucose synthesis has been suggested from experiments (*9*) in which L-sorbose-6-C^{14} yields glucose labeled mainly in C-1 and C-6

It is of interest that a similar isotopic pattern was found in sucrose-derived glucose from a ripening strawberry stem-fed with L-ascorbic acid-6-C^{14} as that obtained in glycogen isolated from rats injected with diketogulonic acid-6-C^{14} (Table IV). These findings suggest the possibility of similar pathways for L-ascorbic acid catabolism in plants and animals.

VI. L-Ascorbic Acid, an Intermediate in Carbohydrate Metabolism

Evidence has been presented for the conversion of glucose to L-ascorbic acid (Section II), as well as for its transformation back to glucose (Section V) Thus it now appears that L-ascorbic acid may be considered an

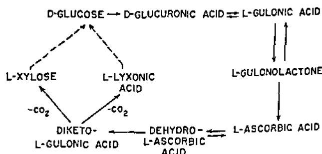


FIG 2 L-Ascorbic acid shown as an intermediate in a cyclic pathway of glucose metabolism in animals

intermediate in carbohydrate metabolism as shown by the cyclic pathway in Fig 2. Man, monkey, and guinea pig are unable to carry out the conversion of L-gulonolactone to L-ascorbic acid (Section III) which is

presumably the result of a gene-controlled enzyme deficiency (*50*). Consequently, scurvy in these species may be considered to arise from a defect in carbohydrate metabolism (*51*)

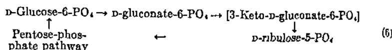
Loewus and co-workers (*34*) have recently postulated a role for L-ascorbic acid as an intermediate in carbohydrate metabolism in plants This was pointed out by the rapid conversion of C^{14} from L-ascorbic acid-6-C^{14} into the carbohydrate pool of the young grape leaf Mature tissues such as the strawberry fruit continue to form L-ascorbic acid but are no longer able to metabolize the compound rapidly, resulting in its accumulation It should be recalled that glucose is converted to L-ascorbic acid in plants by a pathway different from that given in Fig 2.

VII. Glucuronic Acid Pathway of Glucose Metabolism

In recent years a new route of glucose metabolism, the glucuronic acid pathway, has been proposed based on studies (*15, 52, 53*) of the biosyntheses of L-ascorbic acid and L-xylulose (Fig 3) According to this scheme L-gulonic acid serves as a precursor for L-xylulose (*19, 27, 53*) as well as for L-ascorbic acid All the animals studied can metabolize L-gulonic acid via L-xylulose, but only primates and the guinea pig lack the ability to convert L-gulonic acid to L-ascorbic acid (Section III)

Recent studies have provided experimental support for the participation of 3-keto-L-gulonic acid as an intermediate in the formation of L-xylulose by a DPN-dependent enzyme in the soluble fraction of kidney (*54*) Touster *et al* (*55, 56*) have demonstrated an enzyme system in liver capable of reversibly reducing both L-xylulose and D-xylulose to a common intermediate, xylitol, thereby providing a mechanism for the interconversion of the stereoisomers of this ketopentose The subsequent finding by Hickman and Ashwell (*57*) of a specific liver kinase capable of forming D-xylulose-5-PO_4 from the free sugar indicated that mammalian tissues possessed the complete enzymic structure necessary to carry out the conversion of L-xylulose to glucose via the pentose cycle (*58, 59*) Evidence for the occurrence of this cyclic pathway in the intact rat and guinea pig has come from the recent findings that labeled D-glucuronolactone (*60*), L-gulonolactone (*49, 60*) and xylitol (*52*) are converted to liver glycogen in accordance with this scheme

The glucuronic acid pathway represents a new oxidative pathway of glucose metabolism in animals It differs from the hexose monophosphate shunt (*35*), reaction (6), in that glucose is oxidized at C-6 to form D-glucuronic acid instead of at C-1 to form D-gluconic acid However, both pathways channel glucose into the same pentose pool.



The glucuronic acid pathway is of importance for the synthesis of L-ascorbic acid in animals other than primates and the guinea pig It is

FIG 3 The glucuronic acid pathway of glucose metabolism in animals The asterisks denote the fate of C-1 of glucose in the scheme.

of considerable interest that Loewus and co-workers (34) have recently proposed that the hexose monophosphate shunt may be involved in the formation of L-ascorbic acid in plants as shown in Fig. 1. Although there is considerable evidence that the hexose monophosphate shunt is involved

in the synthesis of D-ribose, recent studies by Hiatt and Lareau (*61*) indicate that the glucuronic acid pathway plays little or no role in D-ribose production from glucose. The glucuronic acid pathway also accounts for the origin of L-xylulose, the sugar excreted by human subjects with essential pentosuria.

Various drugs markedly increase the rate at which glucose is metabolized via the glucuronic acid pathway. For example, administration of barbital and Chloretone to rats leads to a marked increase in the conversion of glucose to free D-glucuronic acid (*15*), L-gulonic acid (*14*), and L-ascorbic acid (*15*). This effect on L-ascorbic acid biosynthesis in the rat is shown by many drugs including various barbiturates, aminopyrine, and antipyrine (*62*), but the mechanisms involved are not known. It is of considerable interest that in 1935 Enklewitz and Lasker (*63*) found that two of these drugs, aminopyrine and antipyrine, markedly increased the urinary excretion of L-xylulose in patients with essential pentosuria. It is now possible to explain their observation in terms of the scheme in Fig. 3. Administration of these drugs would be expected to increase the formation of L-xylulose from D-glucose. Since the pentosuric patient is not able to metabolize L-xylulose (*64*), the pentose would consequently be excreted in urine.

VIII. Metabolism of D-Ascorbic Acid

Dayton and Burns (*65*) have compared the metabolism of D-ascorbic acid-1-C^{14} and L-ascorbic acid-1-C^{14} in guinea pigs and rats. D-Ascorbic acid was oxidized appreciably to CO_2, and was distributed in tissues very much like L-ascorbic acid. However, D-ascorbic acid was excreted by the kidney at a considerably more rapid rate than L-ascorbic acid thereby suggesting an explanation for the reported lack of vitamin C activity of the D-isomer in guinea pigs (*66*).

In order to test this hypothesis, bioassay experiments were carried out in guinea pigs under conditions in which essentially comparable tissue concentrations of D-ascorbic acid and L-ascorbic acid were maintained (*67*). The results obtained showed that D-ascorbic acid was able to replace some of the activities of L-ascorbic acid when administered in relatively small doses under the conditions of these experiments. For instance, D-ascorbic acid could maintain the weight and prolong the survival time of scorbutic guinea pigs but it was unable to prevent joint hemorrhages. The most striking effect of D-ascorbic acid was revealed by histochemical studies of the teeth. The dentin produced in the animals receiving D-ascorbic acid was normal in morphology and staining properties. However, the vitamin C effect of D-ascorbic acid was not complete

in that the predentin formed in these animals was still abnormal in its staining properties

The results of these experiments furnish further evidence for multiple actions of vitamin C. those which are specific for only L-ascorbic acid, and those that are nonspecific in which D-ascorbic acid and presumably other compounds with the same redox properties can substitute. The separation of the biological activity of vitamin C has also been indicated by studies with D-araboascorbic acid in guinea pigs (*68*) and with D-ascorbic acid in monkeys (*69*) One of the best examples of a nonspecific role for the vitamin has come from studies of tyrosine metabolism in which L-ascorbic acid can be replaced by various other structurally unrelated compounds that are readily susceptible to oxidation and reduction (*70, 71*). It is possible that further work with D-ascorbic acid or other analogs of the vitamin in guinea pigs may throw light on the nature of these nonspecific functions.

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Metabolism of Phosphatides*

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I. Anabolism

A. General

When a suitable isotopically labeled precursor is administered to an animal, the label is incorporated into the phosphatides of most tissues. This is true for a wide variety of precursors, e g inorganic P labeled with P^{32}, fatty acid labeled with deuterium, C^{14}, I^{131}, or elaidic acid; acetate labeled with deuterium or C^{14}; glycerol labeled with C^{14}, nitrogen-containing bases, such as choline, ethanolamine, or serine, labeled either with C^{14} or with N^{15}; inositol labeled with C^{14} or tritium Similar labeling experiments carried out *in vitro* with tissue slices indicate that for most tissues the phosphatides are formed *in situ* from the appropriate precur-

* Special abbreviations employed: α-GP, α-glycerophosphate, PC, phosphorylcholine, GPC, glycerylphosphorylcholine, CTP, cytidine 5'-triphosphate, CMP-PC, cytidine diphosphate choline, PE, phosphorylethanolamine, GPE, glycerylphosphorylethanolamine, CMP-P-dig, cytidine diphosphate D-α, β-diglyceride

sors [see Chaikoff (*1*), Hevesy (*2*), Chaikoff and Zilversmit (*3*), Wittcoff (*4*), Deuel (*5*), Dawson (*6*), and Rossiter (*7*) for references]

These isotope experiments prompted considerable speculation as to the possible metabolic reactions that might occur in the building up of the phosphatide molecule. However, they gave little information as to which of a number of theoretically possible metabolic pathways is the correct one. Recently, largely as a result of the most notable contributions of Kennedy (*8*, *9*), a certain amount has been learned concerning the reactions leading to the formation of glycerophosphatides, particularly to the formation of lecithin (I) in chicken liver. Similar pathways are operative in brain (*7*, *10*) and probably in most other tissues.

In the present chapter no mention will be made of the formation of the precursors of the phosphatides, e g glycerol, the fatty acids, choline, ethanolamine, serine, and inositol. The biogenesis of these substances is considered elsewhere in this volume

Recent reviews on the biological formation of phosphatides are those of Kennedy (*8*, *9*) and Rossiter and Strickland (*11*)

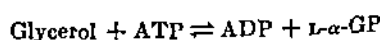
B LECITHIN

1. *Formation of Phosphatidic Acid*

Early *in vivo* experiments by Zilversmit *et al.* (*12*) and by Popják and Muir (*13*) suggested the possibility that α-glycerophosphate (α-GP) might be a precursor of tissue glycerophosphatides, a suggestion that was confirmed by subsequent *in vitro* experiments with liver enzyme preparations (*14*) and mitochondria (*15*).

The α-GP could arise from the glycolysis intermediate, dihydroxyacetone phosphate, by the action of L-α-GP dehydrogenase Two L-α-GP dehydrogenases are known· (a) a particulate enzyme, studied by Green (*16*), Tung *et al* (*17*), and Ringler and Singer (*18*), which is not DPN-linked and occurs principally in brain tissue, and (b) a soluble enzyme, studied by von Euler and colleagues (*19*) and crystallized by Baranowski (*20*) and Young and Pace (*21*), which requires DPN and occurs principally in muscle tissue. Similar L-α-GP dehydrogenases are also found in insects [see Estabrook and Sacktor (*22*), for references] Both of the enzymes are specific for the L-α configuration of GP.

Alternatively, the GP could arise from the phosphorylation of glycerol by the transfer of phosphate from ATP:



$$\text{Glycerol} + \text{ATP} \rightleftharpoons \text{ADP} + \text{L-}\alpha\text{-GP} \qquad (1)$$

Such a reaction was demonstrated in kidney extracts by Kalckar (*23*) and in *Propionibacterium pentosaceum* by Barker and Lipmann (*24*). The

sors [see Chaikoff (*1*), Hevesy (*2*), Chaikoff and Zilversmit (*3*), Wittcoff (*4*), Deuel (*5*), Dawson (*6*), and Rossiter (*7*) for references].

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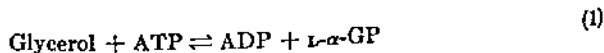
B. Lecithin

1. *Formation of Phosphatidic Acid*

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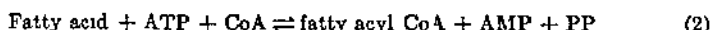
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reaction is catalyzed by the enzyme glycerokinase, which was purified by Bublitz and Kennedy (*25*) and obtained in crystalline form by Wieland and Suyter (*26*). Like the GP formed by the action of the GP-dehydrogenases mentioned above, the GP formed by reaction (1) has the L-α configuraton (*23, 25*)

When inorganic P^{32} is added to mitochondria (*15, 27, 28*) or to homogenates (*27, 29, 30*) from liver or brain, a large percentage of the radioactivity may be recovered from a fraction that has many of the properties of a phosphatidic acid (II) Only small amounts are recovered from lecithin (I), phosphatidyl ethanolamine (III), or phosphatidyl serine (IV). Similarly when α-GP^{32} is the source of radioactivity, Kennedy (*15*) and Kornberg and Pricer (*14*), using liver preparations, and McMurray *et al* (*27*), using brain preparations, found that almost all of the radioactivity appears in phosphatidic acid This incorporation of α-GP into phosphatidic acid is stimulated by the addition to the reaction mixture of coenzyme A (CoA), long-chain fatty acids and ATP (*27, 31*)

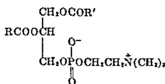
Kornberg and Pricer (*32*) made the important discovery that guinea pig liver contains an enzyme system capable of catalyzing the activation of long-chain fatty acids (up to C_{22}), with the formation of a thioester of CoA



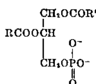
$$\text{Fatty acid} + \text{ATP} + \text{CoA} \rightleftharpoons \text{fatty acyl CoA} + \text{AMP} + \text{PP} \quad (2)$$

With palmitic acid as substrate, palmityl CoA was isolated from the reaction mixture

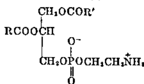
The work of Berg (*33*) indicates that the activation of acetate by



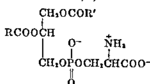
L-α-Lecithin
(L-α-Phosphatidyl choline)
(I)



L-α-Phosphatidic acid
(II)



L-α-Phosphatidyl
ethanolamine
(III)

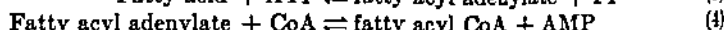


L-α-Phosphatidyl
serine
(IV)

ATP and CoA takes place in two steps. First, ATP and acetate react to form acetyl adenylate and PP. In the presence of CoA the acetyl adenylate forms acetyl CoA and AMP. Jencks and Lipmann (*34*) provided evidence that with an enzyme preparation from hog liver particles, longer chain fatty acids (up to C_{12}) also are activated in two steps, the former not involving CoA. Reaction (2) might therefore be written:



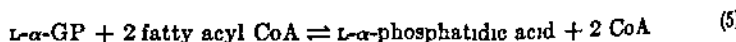
$$\text{Fatty acid} + \text{ATP} \rightleftharpoons \text{fatty acyl adenylate} + \text{PP} \quad (3)$$



$$\text{Fatty acyl adenylate} + \text{CoA} \rightleftharpoons \text{fatty acyl CoA} + \text{AMP} \quad (4)$$

Recently Vignais *et al.* (*35*) reported that brain tissue contains an enzyme capable of activating palmitic acid with the formation of palmityl CoA, according to Eq. (2). Subsequently the same workers provided evidence that both reaction (3) and r... (4) take place in brain and liver tissue, indicating that the mec
chain fatty acids (C_{16}) is similar to
and for acids of intermediate chain length (*36*).

Kornberg and Pricer (*37*) also demonstrated that liver contains a second enzyme system capable of esterifying L-α-GP with fatty acyl CoA to form L-α-phosphatidic acid (II):



$$\text{L-}\alpha\text{-GP} + 2\ \text{fatty acyl CoA} \rightleftharpoons \text{L-}\alpha\text{-phosphatidic acid} + 2\ \text{CoA} \quad (5)$$

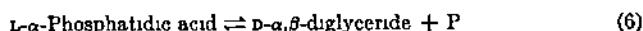
This reaction was studied further by Stansly (*38*). The observation that the addition of CoA greatly accelerates the incorporation of α-GP[32] (*27, 31*), C^{14}-labeled α-GP (*11*), and C^{14}-labeled palmitic acid (*39*) into the phosphatide of tissue preparations, suggests that reactions (2) and (5) are proceeding. The finding that a coupled energy source is required for these incorporations (*15, 27, 39*) ... evidence in favor of this
view, since reaction (2) requires AT
enzyme system catalyzing reaction
into the phosphatidic acid of cell-free tissue preparations

2. *Phosphatidic Acid Phosphatase*

It was stated above that in many cell-free tissue preparations the radioactivity of α-GP[32] is incorporated into phosphatidic acid (II) rather than into the classic glycerophosphatides (I, III, IV). This finding raises the question of the role of phosphatidic acid in the synthesis of glycerophosphatides

Smith *et al* (*40*) showed that phosphatidic acids may give rise to D-α,β-diglycerides (V), which Kennedy and Weiss have shown to be precursors both of glycerophosphatide (*41*) and of triglyceride (*42*)

L-α-phosphatidic acid is dephosphorylated in a number of animal tissue preparations by the enzyme phosphatidic acid phosphatase (*11, 40*):



L-α-Phosphatidic acid ⇌ D-α,β-diglyceride + P (6)

Phosphatidic acid phosphatase enzyme is inhibited by magnesium ions This inhibition probably accounts for the accumulation of phosphatidic acid (*14, 15, 27, 29*) or a "phosphatidic acid-like" compound (*28, 30*) in many tissue preparations, even although only small amounts of phosphatidic acid may be present in the same tissue from freshly killed animals (*43–45*) Recently Hokin and Hokin (*46*) have produced further evidence that phosphatidic acid does occur in animal tissues

The failure to detect any radioactivity in lecithin when α-GP^{32} is added to metabolizing tissue preparations is thus explained The radioactivity is incorporated into phosphatidic acid and is then lost from the labeled phosphatidic acid as inorganic P^{32} by reaction (6) This is in sharp contrast to what happens in similar experiments with C^{14}-labeled α-GP When the glycerol portion of α-GP is labeled, considerable radioactivity may be recovered from lecithin (*11*) The mechanism of this incorporation is discussed below.

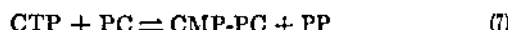
3 *Formation of Lecithin from Phosphorylcholine*

The early *in vivo* experiments of Riley (*47*) with phosphorylcholine (PC) gave little support to the supposition that this substance might be an intermediate in the biosynthesis of lecithin However, interest in PC was renewed when Kornberg and Pricer (*31*) showed that PC doubly labeled with C^{14} and P^{32} is incorporated, as a unit, into the lipids of a rat liver preparation Subsequently, Rodbell and Hanahan (*48*) and McMurray *et al* (*27*) showed that $P^{32}C$ is incorporated into the lecithin of liver and brain preparations The same is true of C^{14}-labeled PC (*11, 49*). The earlier claim that choline-C^{14} is incorporated more rapidly into lecithin than C^{14}-labeled PC has not been substantiated (*49*).

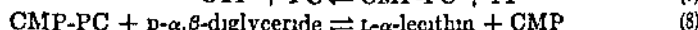
That PC, but not glycerylphosphorylcholine (GPC), can be a precursor of lecithin is shown by the isotope experiments of Dawson (*50, 51*). After the injection of inorganic P^{32} into rats the specific activity of the free PC of the liver is considerably greater than that of the lecithin (*51*). On the other hand, the specific activity-time curves of lecithin and GPC satisfy the criteria of Zilversmit *et al* (*52*) for an immediate precursor-product relationship (*50*). From these experiments it can be concluded that PC is a possible precursor of lecithin and that GPC is a probable degradation product.

A great stimulus to the study of the biosynthesis of phosphatides was

the important finding of Kennedy and Weiss (*41, 53*) that cytidine 5′-triphosphate (CTP) is necessary for the incorporation of $P^{32}C$ into lecithin. The requirement is specific for CTP, none of a number of other nucleoside 5′-triphosphates being active (*27, 41*). Kennedy and Weiss (*41*) showed that lecithin is formed according to the following reactions:

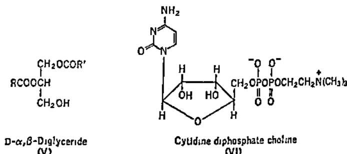


$$\text{CTP} + \text{PC} \rightleftharpoons \text{CMP-PC} + \text{PP} \quad (7)$$



$$\text{CMP-PC} + \text{D-}\alpha,\beta\text{-diglyceride} \rightleftharpoons \text{L-}\alpha\text{-lecithin} + \text{CMP} \quad (8)$$

The *formation of cytidine diphosphate choline (CMP-PC)* (VI) in reaction (7) represents a new role for cytidine nucleotides in metabolic processes. In the subsequent reaction [reaction (8)] PC is transferred from CMP-PC (VI) to a D-α,β-diglyceride (V) acceptor, to form L-α-lecithin with the production of cytidine 5′-monophosphate (CMP). The



D-α,β-Diglyceride (V)   Cytidine diphosphate choline (VI)

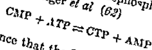
novel intermediate, CMP-PC, occurs widely in animal tissues (*41*), as well as in yeast (*54, 55*). The natural occurrence of deoxycytidine diphosphate choline also has been reported (*56, 57*).

The enzyme catalyzing reaction (7) was called PC-cytidyl transferase by Borkenhagen and Kennedy (*58*), who studied the reaction in some detail. The enzyme, obtained from guinea pig liver, is particle-bound and requires magnesium or manganous ions for full activity. It is specific for CTP and readily catalyzes the reverse reaction, i.e., the pyrophosphorolysis of CMP-PC, with the formation of PC and CTP.

The enzyme catalyzing reaction (8) was called PC-glyceride transferase by Weiss *et al.* (*59*). These workers studied the enzyme in particle preparations from chicken liver and rat liver. The reaction is readily reversible, yielding CMP-PC and D-α,β-diglyceride by a "cytidylolysis" of lecithin.

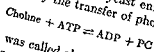
There is good evidence that enzymes catalyzing the PC-cytidyl and glyceride transferase reactions [reactions (7) and (8)] are present in brain (*10, 11, 27, 60*) and seminal vesicle (*61*). It is probable that they occur in many other tissues.

The work of Rodbell and Hanahan (*43*) and Kennedy and Weiss (*41*) with liver preparations and that of McMurray *et al* (*27*) with brain preparations indicate that a coupled energy source is necessary for the incorporation of labeled PC into lecithin This is required for the maintenance of a sufficiently high concentration of CTP to enable reaction (7) to proceed Presumably CTP is formed by the phosphorylation of the CMP, a product of reaction (8), by transphosphorylase enzymes similar to those described by Strominger *et al* (*62*)



$$\text{CMP} + \text{ATP} \rightleftharpoons \text{CTP} + \text{AMP} \qquad (9)$$

There is good evidence that the PC, which is required for the formation of CMP-PC (*54*) by reaction (7), can arise in most tissues Wittenberg and Kornberg (*63*) partially purified a yeast enzyme that catalyzes the phosphorylation of choline by the transfer of phosphate from ATP.



$$\text{Choline} + \text{ATP} \rightleftharpoons \text{ADP} + \text{PC} \qquad (10)$$

The enzyme, which was called choline phosphokinase by Wittenberg and Kornberg (*63*), was said to be present in a number of mammalian tissues A similar enzyme is present in rapeseed (*64*) and in brain and nerve tissue (*65*)

The D-α,β-diglyceride, which is the acceptor of PC from CMP-PC, according to reaction (8), is probably derived from the dephosphorylation of phosphatidic acid, as mentioned above [reaction (6)] This is possibly a major source of D-α,β-diglyceride *in vivo* It has been indicated already that in most energy-yielding tissue preparations, reaction (6) is inhibited by the high concentration of magnesium ions necessary for *in vitro* phosphorylative processes However, the formation of α,β-diglyceride from such a source may be demonstrated by experiments in which either liver (*40*) or brain (*11*) preparations are preincubated with phosphatidic acid in the absence of magnesium ions. Under these conditions the D-α,β-diglyceride formed by the dephosphorylation of the added phosphatidic acid is able to accelerate reaction (8)

Another possible source of α,β-diglyceride in some tissues is triglyceride Diglycerides are derived from triglycerides by the action of lipases (*66*, *67*), although the experiments of Karnovsky and Wolff (*68*) indicate that pancreatic, wheatgerm, and clearing-factor lipase lack stereospecificity

4 *Fatty Acids*

In the foregoing discussion of the biogenesis of lecithin no mention has been made of the well known fact that lecithins from different sources vary considerably in the nature of their constituent fatty acids Hanahan

(69) examined the fatty acids derived from lecithins isolated from the liver of a number of different species In each instance the fatty acids esterified in the α′ position were unsaturated, while those in the β position were saturated The same is true for egg lecithin (70, 71), indicating what Hanahan has called a positional asymmetry of the fatty acids

The experiments of Hanahan and Blomstrand (72a) indicate that as well as a positional asymmetry of the fatty acids in lecithin, there is also a metabolic asymmetry In isotope experiments, saturated fatty acids, in general, exchange with the fatty acids esterified in the β position, whereas unsaturated fatty acids exchange much more readily, and largely with the fatty acids in the α′ position (72a, 72b). Similarly Rhodes (73a) found that if cod-liver oil is fed to a laying hen, the fatty acids esterified in the α′ position become more unsaturated, while the fatty acids in the β position remain for the most part saturated

If the metabolic pathways described above have a general application, this positional configuration must be initiated at the stage where L-α-GP is esterified [reaction (5)] and it must be maintained throughout the D-α,β-diglyceride stage. At the present time the mechanism of reaction (5) is not understood It is probable that the reaction takes place in at least two stages, although little is known of the details, or of the fatty acid specificity of each esterification

With the wider application of the technique of gas chromatography, it is possible that in the near future more information will become available concerning the metabolism of the fatty acids of lecithin and the other phosphatides

5. *Comment*

The pathways described above for the formation of lecithin in animal tissues are summarized in Fig 1. It should be stressed that, although there is good evidence for the occurrence of all the reactions represented in the figure, it is probable that such a sequence of reactions represents a gross oversimplification It is by no means certain that each of a wide variety of lecithins that are known to occur in many different parts of any given cell in any one tissue, are formed by the same series of metabolic reactions For example, Hokin and Hokin (73b) have shown that phosphatidic acid may be formed by the transfer of a phosphate group from ATP to D-α,β-diglyceride in the presence of the enzyme diglyceride kinase, as well as by the esterification of L-α-GP. Another example is the preliminary report of Bremer and Greenberg (73c), indicating the possibility that lecithin may be formed by the direct transfer of the methyl group of methionine to phosphatidyl ethanolamine Experiments with methionine (Me-C^{14}) suggest that phosphatidyl ethanolamine itself,

rather than any of the known intermediates in the synthesis of phosphatidyl ethanolamine (see below), is the acceptor of the labeled methyl group. There is good reason to suppose that in the future other alternative metabolic pathways will be discovered

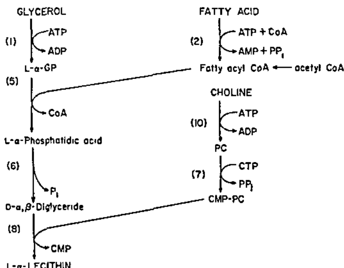
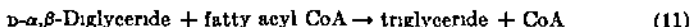


FIG 1 Proposed scheme for formation of lecithin, after Kennedy (*41, 63*) Figures in parentheses refer to equations in text

Another point worthy of notice is the finding by Weiss and Kennedy (*42*) that D-α,β-diglycerides are capable of esterification with fatty acyl CoA to form triglycerides·


D-α,β-Diglyceride + fatty acyl CoA → triglyceride + CoA (11)

Evidence in favor of the supposition that D-α,β-diglyceride derived from the dephosphorylation of phosphatidic acid is a precursor of tissue triglyceride, is the finding of Stein and Shapiro (*74*) that in cell-free preparations from liver C^{14}-labeled α-GP is incorporated into the triglyceride much more readily than C^{14}-labeled glycerol It will be recalled that the same was true for the labeling of phosphatidic acid

Figure 2 shows these two possible metabolic pathways for D-α,β-diglyceride, which may be the precursor of either lecithin (or other glycerophosphatides) or triglyceride.

Lands (*75*) showed that in slices of lung tissue triglycerides and phosphatides are labeled to a similar degree from glycerol-C^{14}, but not

from acetate-C^{14} As Lands (*75*) has pointed out, such an observation is capable of many interpretations. It seems clear, however, that lecithin and triglyceride are not derived from a common pool of D-α,β-diglyceride, nor probably are lecithin and phosphatidyl ethanolamine (*73a*)

Such observations in no way invalidate the proposed pathways for the formation of glycerophosphatide and triglyceride, but they do indicate that the respective D-α,β-diglyceride precursors are not in isotope equilibrium It is possible that different lipids are formed at different sites within the cell, or that the constituent fatty acids of a diglyceride

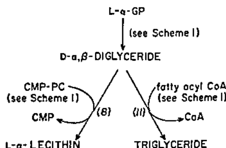


FIG 2 Proposed scheme for formation of either L-α-lecithin or triglyceride from D-α,β-diglyceride, after Weiss and Kennedy (*42*) Figures in parentheses refer to equations in text For Scheme 1 see Fig 6

destined to become a triglyceride confer to the diglyceride molecule a specificity different from that of a diglyceride destined to become a glycerophosphatide.

C PHOSPHATIDYL ETHANOLAMINE

The early *in vivo* experiments of Chargaff and Keston (*76*) with phosphorylethanolamine (PE), like those of Riley (*47*) with PC, suggested that PE is not an intermediate in the biosynthesis of phosphatidyl ethanolamine (III) However, the work of Kennedy described above on the synthesis of lecithin led to a reinvestigation of the problem

Dawson (*50*), in isotope experiments similar to those already described for lecithin, showed that glycerylphosphorylethanolamine (GPE) cannot be a precursor of phosphatidyl ethanolamine and that GPE is probably a product of the degradation of phosphatidyl ethanolamine.

The role of PE in the biosynthesis of phosphatidylethanolamine was established when Borkenhagen and Kennedy (*58*) showed that guinea pig liver contains the enzyme PE-cytidyl transferase This enzyme catalyzes the formation of cytidine diphosphate ethanolamine by a reaction analogous to reaction (7) Kennedy and Weiss (*41*) also showed that

chicken liver contains PE-glyceride transferase, an enzyme that catalyzes the transfer of PE from cytidine diphosphate ethanolamine to D-α,β-diglyceride by a reaction analagous to reaction (8) Rossiter and Strickland (*11*) showed that similar enzymes are present in brain tissue

It would appear that the sequence of reactions leading to the formation of phosphatidyl ethanolamine is not greatly different from that leading to the formation of lecithin

Presumably there is an adequate supply of the necessary precursors in most tissues The formation of D-α,β-diglyceride has been described already and there is good isotope evidence that mammalian tissues are capable of phosphorylating ethanolamine (*77*) Also the yeast enzyme of Wittenberg and Kornberg (*63*), that has the ability to catalyze the transfer of phosphate from ATP to choline, is known to phosphorylate ethanolamine, although the choline kinase of rapeseed, recently studied by Ramasarma and Wetter (*64*), is inactive

D PHOSPHATIDYL SERINE

Isotope experiments indicate that phosphatidyl serine (IV) may be labeled from acetate-C^{14}, glycerol-C^{14}, and serine-C^{14} [Pritchard (*78*)] However, little is known of the metabolic pathways involved

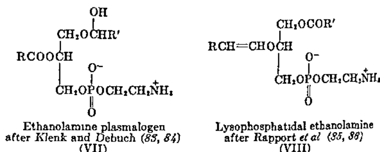
By analogy with lecithin and phosphatidyl ethanolamine, it is possible that phosphoserine may be an intermediate in the synthesis of phosphatidyl serine However, in both plant (*64*) and animal (*79*) tissues no evidence for the phosphorylation of serine was reported, although it has been shown that liver tissue contains a powerful phosphoserine phosphatase (*80, 81*) On the other hand, Ichihara and Greenberg (*79*) presented evidence that phosphoserine is formed in animal tissue from 3-phosphoglyceric acid by way of 3-phosphohydroxypyruvic acid

The experiments of Hubscher *et al* (*82*) on the incorporation of C^{14}-labeled serine into the phosphatidyl serine of rat liver mitochondria indicate that CMP, but not CTP, and CoA are necessary for maximum incorporation The authors suggest that the synthesis of phosphatidyl serine may be brought about by a metabolic pathway different from that described above for lecithin and phosphatidyl ethanolamine No experiments with labeled phosphoserine were reported

E PLASMALOGEN

Klenk and Debuch (*83, 84*) favored a hemiacetal form (VII) as one of several possible structures for tissue plasmalogen Subsequently Rapport and colleagues (*85, 86*) provided evidence for an α′,β′-unsatu-

rated ether structure (VIII) The latter workers suggested that ethanolamine-containing plasmalogen be called phosphatidal ethanolamine. More recent work has tended to substantiate formula VIII [Gray (87), Debuch (88)], although Marinetti *et al.* (89) recently have indicated that in at least some of the plasmalogen from pig heart, the unsaturated ether side chain is attached to the α' rather than the β carbon atom of the glycerol



Ethanolamine plasmalogen after Klenk and Debuch (83, 84) (VII)

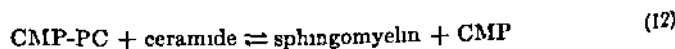
Lysophosphatidal ethanolamine after Rapport *et al* (85, 86) (VIII)

In addition to ethanolamine plasmalogen it is now known that both choline (90–92) and serine (93, 94) may replace ethanolamine in the structure represented by (VII) or (VIII).

At the present time little is known concerning the biosynthesis of plasmalogen Recently Kiyasu (95) reported that rat liver contains an enzyme that catalyzes the formation of choline-containing plasmalogen (cf VIII) from C^{14}-labeled CMP-PC and "plasmalogen diglyceride" The latter is the plasmalogen analog of D-α,β-diglyceride (V), prepared by the action of phospholipase C on choline-containing plasmalogen of beef heart Presumably the reaction is catalyzed by a PC-transferase similar to the PC-glyceride transferase responsible for reaction (8).

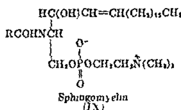
F SPHINGOMYELIN

Sribney and Kennedy (96) showed that chicken liver contains an enzyme that is able to promote the formation of sphingomyelin (IX) from ceramide (*N*-acylsphingosine) (X), according to the reaction·

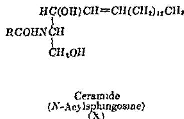


$$\text{CMP-PC} + \text{ceramide} \rightleftharpoons \text{sphingomyelin} + \text{CMP} \quad (12)$$

This reaction, catalyzed by the enzyme PC-ceramide transferase, is analogous to reaction (8), catalyzed by PC-glyceride transferase. In reaction (12) *N*-acylsphingosine (X) rather than D-α,β-diglyceride (V) is the acceptor of the PC transferred from the CMP-PC. A similar reaction occurs in brain tissue (10, 11)



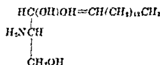
Sphingomyelin (IX)



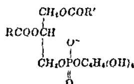
Ceramide (N-Acylsphingosine) (X)

In a second report Sribney and Kennedy (*97*) referred to the substance that accepts PC from CMP-PC in reaction (12) as "active ceramide" It was shown that the sphingosine (XI) moiety of the "active ceramide" was *threo*-1,3-dihydroxy-2-amino-4-*trans*-octadecene, rather than the corresponding *erythro* compound, which is a constituent of many naturally occurring sphingolipids (*98*)

There is now some information concerning the nature of the metabolic pathway leading to the biosynthesis of sphingosine (XI), one of the precursors of sphingomyelin The early *in vivo* experiments of Zabin and Mead (*99, 100*) and Sprinson and Coulon (*101*) indicated that carbon atoms 1 and 2 and the nitrogen atom of sphingosine are derived from the carbon atoms 3 and 2 and the nitrogen of serine. The remaining carbon atoms, 3–18, are derived from acetyl CoA and were thought to enter the sphingosine molecule by way of a long-chain 16-carbon intermediate.



Sphingosine (XI)



L-α-Phosphatidyl inositol (XII)

These findings were substantiated by the *in vitro* experiments of Brady and Koval (*102*), who showed that palmityl CoA may be a precursor of carbon atoms 3–18 of sphingosine and that the addition of TPNH to the reaction mixture is necessary when serine and palmityl CoA are the precursors Magnesium ions and pyridoxal phosphate also are required Zabin (*103*) described a rat brain system, with similar coenzyme requirements, that formed ceramide from serine and palmityl CoA

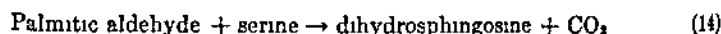
Recently Brady *et al* (*104*) have shown that when sphingosine is formed from palmityl CoA and serine, the first stage in the over-all reaction is the formation of palmitic aldehyde from palmityl CoA. The

reduction, which requires TPNH, is catalyzed by an enzyme present in rat brain (*102*):



$$\text{Palmityl CoA} + \text{TPNH} + H^+ \rightleftharpoons \text{palmitic aldehyde} + \text{CoA} + \text{TPN}^+ \quad (13)$$

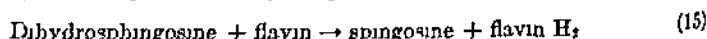
The palmitic aldehyde condenses with serine to form dihydrosphingosine and CO_2



$$\text{Palmitic aldehyde} + \text{serine} \rightarrow \text{dihydrosphingosine} + CO_2 \quad (14)$$

Reaction (14) requires pyridoxal phosphate and manganous ions Brady *et al* (*104*) produced evidence that a Schiff base-metal complex is formed with serine, pyridoxal phosphate, and manganous ions It is suggested that the formation of such a complex results in the activation of the methylene group at carbon atom 2 of the serine The resonance-stabilized carbanion so formed can then participate in the carbon-to-carbon addition reaction with palmitic aldehyde to form dihydrosphingosine and CO_2. This reaction bears a strong resemblance to an aldol condensation of the Knoevenagel type and may represent a novel mechanism for the elongation of carbon chains in biological systems

It is suggested further that the dihydrosphingosine is converted to sphingosine by a flavoprotein enzyme present in brain tissue (*102*, *104*):



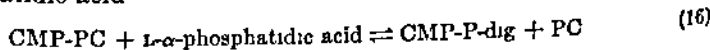
$$\text{Dihydrosphingosine} + \text{flavin} \rightarrow \text{spingosine} + \text{flavin } H_2 \quad (15)$$

Presumably the sphingosine is esterified by fatty acyl CoA before it participates in reaction (12) The reactions leading to the formation of sphingomyelin are summarized in Fig 3

G. Inositol Phosphatide

Of recent years considerable interest has been shown in the inositol phosphatides A phosphatidyl inositol (XII) has been isolated from liver (*105–107*), heart (*108*, *109*), and several different plant sources (*110*, *111*), although it is probable that the phosphatidyl inositol isolated from plants is derived from a more complex inositol phosphatide (*112*, *113*)

Recently Agranoff *et al* (*114*) showed that *myo*-inositol is incorporated into the inositol phosphatide of a particulate preparation from guinea-pig kidney mitochondria. The incorporation is greatly stimulated by the addition of either CMP-PC or CMP, but not by the addition of CTP or CDP. The incorporation is also greatly stimulated by the addition of phosphatidic acid, but not by the addition of D-α,β-diglyceride It is suggested that a lipid-soluble intermediate, cytidine diphosphate D-α,β-diglyceride (CMP-P-dig), is formed from the reaction between CMP-PC and phosphatidic acid·



$$\text{CMP-PC} + \text{L-}\alpha\text{-phosphatidic acid} \rightleftharpoons \text{CMP-P-dig} + \text{PC} \quad (16)$$

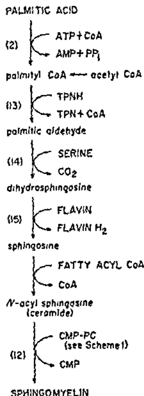
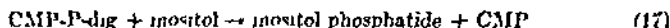


FIG 3 *Proposed scheme for formation of sphingomyelin, after Brady, Formica, and Koval (104) and Sribney and Kennedy (97) Figures in parentheses refer to equations in text For Scheme 1 see Fig 6*

The CMP-P-dig then reacts with the hydroxyl group of inositol in a pyrophosphorylytic reaction analogous to the PC-glyceride transferase reaction between CMP-PC and the hydroxyl group of D-α,β-diglyceride (reaction 8)



$$\text{CMP-P-dig} + \text{inositol} \rightarrow \text{inositol phosphatide} + \text{CMP} \qquad (17)$$

The stimulating effect of CMP can be attributed to its conversion to CMP-PC by a reversal of reaction (8)

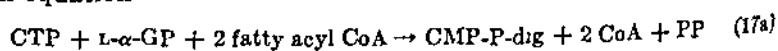
It should be noted that in this scheme inositol is not phosphorylated before it is incorporated into the inositol phosphatide On the other hand, the experiments of Hokin and Hokin on the stimulating effect of acetylcholine on the incorporation of inorganic P^{32} and tritium-labeled inositol into the inositol phosphatides of both pancreas slices (115) and brain

slices (*116*) can be interpreted as favoring the view that the inositol is phosphorylated before it is incorporated into the phosphatide. When either *inorganic* P^{32} *or tritium-labeled inositol is the source of the radio*activity, the labeling of the inositol lipid is increased to a similar extent in the presence of acetylcholine.

Agranoff *et al.* (*114*) suggest that the inositol phosphatide of kidney is a monophosphoinositide, probably similar to the phosphatidyl inositol (XII) prepared from liver and plant sources. In contrast, Folch (*117*) showed that calf brain contains a diphosphoinositide, which on hydrolysis yields fatty acid, glycerol, and inositol metadiphosphate, in equimolecular proportions. However, Hokin and Hokin (*116*) have recently reported the presence of a metabolically active monophosphoinositide in guinea pig brain slices

McMurray *et al* (*27*) showed that the addition of CTP greatly stimulates the incorporation of inorganic P^{32} into the inositol phosphatide of brain homogenates and mitochondria. Subsequently Thompson *et al* (*118a*) showed that C^{14}-labeled and tritium-labeled inositol is incor-

Cytidine nucleotides also stimulate the incorporation, but in the brain preparation, unlike the kidney preparation, CTP and to a lesser extent CDP are superior to CMP and CMP-PC (*118a*) The same is true for the conversion of L-α-GP^{32} to inositol monophosphatide in guinea pig liver preparations (*118b*). More recently Paulus and Kennedy (*118c*) have shown that in enzyme preparations from guinea pig liver, CMP-P-dig is formed from L-α-GP, CoA, and oleic acid, presumably according to the over-all equation



$$\text{CTP} + \text{L-}\alpha\text{-GP} + 2\text{ fatty acyl CoA} \rightarrow \text{CMP-P-dig} + 2\text{ CoA} + \text{PP} \quad (17a)$$

The reaction has not as yet been studied in detail, but it is possible *that CMP-P-dig may be formed from* phosphatidic acid and CTP by a cytidyl transferase reaction analogous to the PC-cytidyl transfer of reaction (7) Paulus and Kennedy (*118c*) also showed that the CMP-P-dig so formed may then react with inositol according to reaction (17).

In kidney, liver, and brain systems cytidine nucleotides and phosphatidic acid are involved in the formation of inositol phosphatide The participation of phosphatidic acid in such a reaction accounts for the high specific activity of the inositol phosphatide relative to that of the classic glycerophosphatides observed when tissue preparations are incubated in the presence of inorganic P^{32} (*27–30*) As described previously the P^{32} of *radioactivity is rapidly incorporated into* phosphatidic acid, the P^{32} of

which is then transferred with the glycerol and fatty acid to the inositol phosphatides It will be recalled that in the formation of the classic glycerophosphatides the P^{32} that is incorporated into phosphatidic acid is lost as inorganic P^{32} by the action of phosphatidic acid phosphatase [reaction (6)]

The suggested reactions leading to the formation of inositol phosphatide are summarized in Fig 4

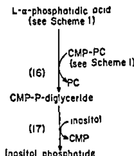


FIG 4 Proposed scheme for formation of inositol phosphatide, after Agranoff, Bradley, and Brady (*114*) Figures in parentheses refer to equations in text For Scheme 1 see Fig 6

II. Catabolism

A General

Enzymes capable of degrading phosphatides are present in snake venoms, plants, and microorganisms It is only recently, however, that the presence of such enzymes in animal tissues has been established beyond question In much of the earlier work bacterial contamination was not controlled

Bókay (*119*) in 1877 and later Mayer-Karlsbad (*120*) reported that

tissues contain enzymes capable of degrading phosphatides.

The enzymes that hydrolyze the glycerophosphatides have been referred to variously as lecithinases, lecithases, lecitholipases, phosphatidolipases, phosphatidases, and phospholipases The term *phospholipase* will be used here, for no better reason than that it occurs widely in the literature

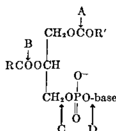
Four classes of phospholipase are known They were designated A, B, C, and D by Contardi and Ercoli (*122*) Phospholipase A is now known to liberate the fatty acid from the α′ position of the glycerophos-

phatide molecule, with the formation of the corresponding lysoglycerophosphatide. Phospholipase B removes the remaining fatty acid from the β position of the lysophosphatide, with the formation of a diester of phosphoric acid with glycerol and the appropriate nitrogen-containing base.

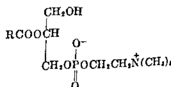
Unfortunately, there is much confusion in the literature concerning the terminology for phospholipase C and phospholipase D. In 1933 Contardi and Ercoli (*122*), who were responsible for the introduction of the alphabetical nomenclature, suggested the term phospholipase C (or rather lecithinase C) for the enzyme, at the time still to be discovered, that detaches the nitrogen-containing base from the glycerophosphatide with the formation of a phosphatidic acid. When such an enzyme was discovered by Hanahan and Chaikoff (*123*) in 1947, it was referred to as phospholipase C, both by Hanahan and Vercamer (*124*) and by Kates (*125*). In the meantime, however, Macfarlane and Knight (*126*) in 1942 had described the presence of an enzyme in *Clostridium welchii* that detaches PC from lecithin with the formation of α,β-diglyceride. They termed this enzyme phospholipase C (or rather, lecithinase C), a logical name for a third phospholipase.

Although Wittcoff (*4*) used the original Contardi and Ercoli (*122*) nomenclature, in more recent reviews [e.g. (*127–129*)] the term phospholipase C is used for the Macfarlane bacterial enzyme and phospholipase D for the Hanahan plant enzyme. This terminology is employed here.

Phospholipase D thus liberates the nitrogenous base from the glycerophosphatides, with the formation of phosphatidic acid, whereas phospholipase C hydrolyzes the ester linkage between the glycerol and the phosphate, with the formation of the appropriate phosphorylated base and α,β-diglyceride. The site of action of the phospholipases is represented in XIII.



Site of action of phospholipases
(XIII)



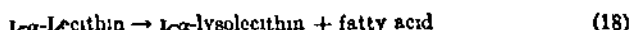
L-α-Lysolecithin
(XIV)

Previous reviews on the phospholipases are those of Belfanti *et al.* (*130*), Ercoli (*131*), Wittcoff (*4*), and Zeller (*127*).

B LECITHIN

1 *Phospholipase A*

Phospholipase A forms lysolecithin (XIV) from lecithin (I) by the removal of the fatty acid esterified in the α' position.


L-α-Lecithin → L-α-lysolecithin + fatty acid (18)

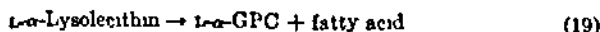
The enzyme is present in the venom of the cobra and many other reptiles (*132–134*) and also in the pancreas (*135*, *136*), salivary glands (*137*), prostate, thymus, and other animal tissues (*138*) and in the spleen of man in certain pathological conditions (*139*) The observations of Francioli (*138*) suggest that the enzyme may occur widely in mammalian tissues It also occurs in plants (*140*) and fungi (*141*) Venom phospholipase A was crystallized by De (*142*) Highly purified preparations have been obtained from a number of sources (*142–144*)

Flexner and Noguchi (*145*) as early as 1902 showed that the enzyme is remarkable in that it is extremely heat stable, a property that has proved useful for the obtaining of preparations free from the activity of contaminating enzymes Another finding of interest is the discovery by Hanahan (*146*) that the activity of the enzyme is enhanced by the presence of moist diethyl ether, a property which phospholipase A shares with other phospholipases

Phospholipase A, which is inactive toward lysolecithin (*134*), is capable of removing both saturated and unsaturated fatty acids from lecithins, although at different rates (*147*, *148*) Hanahan (*149*) and Long and Penny (*150a*) concluded that the fatty acid attached to the α' position on the lecithin molecule is selectively detached, although recently this view has been criticized by Marinetti *et al* (*150b*) Long and Penny (*148*) also showed that the enzyme can remove fatty acid from both natural and synthetic L-α-lecithins, but not from D-α-lecithins, or from β-lecithins

2 *Phospholipase B*

Phospholipase B is responsible for the removal of the remaining fatty acid from lysolecithin with the formation of GPC


L-α-Lysolecithin → L-α-GPC + fatty acid (19)

Contardi and Ercoli (*151*) showed that both rice bran and *Aspergillus oryzae* contain an enzyme capable of rendering the phosphorus of lysolecithin water-soluble Subsequently Fairbairn (*152*) demonstrated the presence of phospholipase B in *Penicillium notatum* The enzyme attacks

lysolecithin, but is inactive towards lecithin. These findings were confirmed by Uziel and Hanahan (*153*), who isolated L-α-GPC from the reaction mixture Uziel and Hanahan (*154*) demonstrated that extracts of *Penicillium notatum* also contain an enzyme that catalyzes the migration of the fatty acid acyl group from the β to the α' position This enzyme, which is particularly active toward saturated lysolecithins, was called lysolecithin migratase.

The first clear-cut illustration of phospholipase B in animal tissues was the preparation by Shapiro (*155*) of a crystalline enzyme from pancreas that removes the fatty acid from lysolecithin. Subsequently Dawson (*156*) demonstrated the presence of a similar enzyme in rat and sheep liver Earlier Schmidt *et al.* (*157*) had isolated GPC from pancreas and had shown that it is formed as the result of the hydrolysis of lecithin according to reactions (18) and (19). This finding suggests that pancreas contains both phospholipase A and phospholipase B, and explains the wide occurrence of GPC in animal tissues (*50, 158*).

This view was strengthened by the experiments of Dawson (*50*), who compared the specific activity of free GPC with that of the lecithin of rat liver at various time intervals after the injection of inorganic P^{32}. From the results he concluded that, unlike PC (*31, 51*), GPC is a degradation product of lecithin rather than a precursor.

More recently, Dawson (*159a*) reported a finding that may be of considerable importance relative to the breakdown of lecithin in animal tissues In rat liver there is a lipid fraction that enables the phospholipase B of *Penicillium notatum* to remove the fatty acids from emulsions of lecithin as well as from lysolecithin This active fraction contains a monophosphoinositide and a polyglycerol phospholipid The addition of these activating lipids causes a reduction in the extent to which lecithin can be extracted from aqueous emulsions by lipid solvents and an *increase in the net negative charge on the lecithin particles* (*159b*) Bangham and Dawson (*159b*) suggest that the electrophoretic charge on the substrate may regulate the activity of the enzyme

3 *Phospholipase C*

There are many references in the literature to preparations having phospholipase C activity However, the first convincing phospholipase C preparation was obtained by Macfarlane and Knight (*126*) in 1941. These workers showed that the toxin of *Clostridium welchii* contains a *component that catalyzes the liberation of PC from lecithin*, with the formation of α,β-diglyceride (V)·

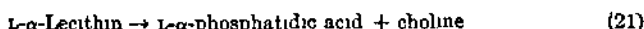

L-α-Lecithin → D-α,β-diglyceride + PC (20)

Macfarlane (*160*) showed that the enzyme is inactive toward phosphatidyl ethanolamine and phosphatidyl serine, so that perhaps it should properly be called a lecithinase C rather than a phospholipase C It is also inactive toward lysolecithin and GPC (*161*) The enzyme occurs in *Clostridium oedematiens* (*162*) and *C. perfringens* (*163*).

There is no great phospholipase C activity in animal tissue It is probable that much of the activity that has been reported in the past was the result of bacterial contamination However, Druzhinina and Kritzman (*164*) claimed that a phospholipase C is present in brain and kidney

4 *Phospholipase D*

Although an enzyme with phospholipase D activity had been postulated for many years, it was not until 1947 that Hanahan and Chaikoff (*123*, *165*) clearly demonstrated the presence in carrots of an enzyme capable of releasing choline from lecithin, with the formation of phosphatidic acid (II)



L-α-Lecithin → L-α-phosphatidic acid + choline (21)

The enzyme is present in carrot (*123*, *165*, *166*), cabbage (*166–168*), sugar beets, spinach (*166*), cottonseed, and other plant sources (*169–171*).

Phospholipase D acts upon glycerophosphatides with the L-α-structure, including many naturally occurring and synthetic lecithins (*125*, *172a*) According to Kates (*125*) the enzyme is inactive toward lysolecithin, GPC, or PC. It is slightly active toward synthetic lecithins with the DL-α- or β-structure (*172a*) It is thus different from the phospholipase A of snake venom, which has a very high degree of specificity for glycerophosphatides with the L-α-configuration However, the enzyme resembles phospholipase B in that it is activated by phosphatidyl inositol (*172b*).

Kates (*166*) reported that for carrot and the leaf of spinach, cabbage, and sugar beet, the enzyme is mainly present in the plastids and that, like phospholipase A, its activity is greatly increased by the addition of diethyl ether However, Davidson and Long (*172a*) showed that there is also a soluble phospholipase C in the cell sap of the Savoy cabbage and several other plant species This enzyme also slowly removes the choline from lysolecithin

Hanahan and Chaikoff (*167*) demonstrated that the isolation of phosphatidic acid from fresh cabbage, first performed by Chibnall and Channon (*173*) in 1927, was achieved because of the presence in the cabbage of a phospholipase D, which degrades the plant glycerophosphatides to phosphatidic acids When the cabbage is treated with steam to inactivate the phospholipase D, the lipid that is isolated still retains the nitrogenous base. Similar findings were reported for carrot (*165*)

5. *GPC-Diesterase*

The early studies of Contardi and Ercoli (*122*) on the enzymic hydrolysis of lecithin suggested that free choline may be liberated. The participation of an enzyme capable of splitting diesters of phosphoric acid was established beyond question by Hayaishi and Kornberg (*174*), who demonstrated the presence of an adaptive GPC-diesterase in *Serratia plymuthica*. The enzyme catalyzes the reaction:



$$\text{L-}\alpha\text{-GPC} \rightarrow \text{L-}\alpha\text{-GP} + \text{choline} \qquad (22)$$

Dawson (*175*) showed that a similar enzyme is present in the liver and other tissues of the rat. The enzyme does not liberate choline from phosphorylcholine, lysolecithin, or lecithin (*175*). Webster *et al.* (*176*) reported the presence of a similar enzyme in rat brain.

6. *Phosphomonoesterases*

Both acid and alkaline phosphomonoesterases capable of hydrolyzing GP are widely distributed in nature. The occurrence, distribution, and properties of these enzymes have been reviewed frequently and will not be discussed further. Such enzymes are able to hydrolyze GP, formed by reaction (22), into glycerol and inorganic phosphate.

Presumably similar enzymes are capable of hydrolyzing PC, for Roche and Bouchilloux (*177*) reported that preparations from liver, prostate, intestine, and kidney are able to liberate choline and inorganic phosphate from PC. The hydrolysis, which also occurs in brain (*178, 179*) is more active at alkaline than at acid pH (*177–179*). However, the enzyme responsible for the hydrolysis of PC may not be of any great significance in the degradation of lecithin in mammalian tissues, for the GPC formed as the result of reaction (19) is broken down into GP and choline by reaction (22), rather than into PC and glycerol.

7. *Comment*

From the foregoing it seems clear that in animal tissues lecithin is broken down first by the action of phospholipase A, which removes the fatty acid attached to the α' carbon to form lysolecithin, and then by the action of phospholipase B, which removes the second fatty acid to form GPC. The GPC is split by GPC-diesterase into choline and GP, which may be degraded further by tissue phosphomonoesterases. Alternatively, the GP may be reesterified to form phosphatidic acid by reaction (5) or it may be converted into dihydroxyacetone phosphate, an

intermediate of glycolysis, by L-α-GP dehydrogenase. The breakdown of lecithin in animal tissues is shown in Fig 5

Such a scheme for the breakdown of lecithin has been postulated for many years, but it is only recently that good evidence has been obtained for the presence in mammalian tissues of each of the necessary enzymes (*156*) Hayaishi and Kornberg (*174*) demonstrated that a similar sequence of reactions occurs in *Serratia plymuthica*

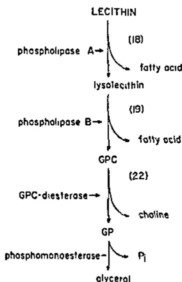


FIG 5 Proposed scheme for hydrolysis of lecithin in animal tissue, after Dawson (*156*)

Although there is good evidence that each of the reactions described above takes place in animal tissues, it cannot be said for certain that these are the only reactions leading to the degradation of lecithin. It is possible that lecithin may be broken down by other pathways For instance reference has been made already to the fact that the PC ...

The CMP-PC could then react with PP by a reversal of reaction (7) to form PC and CTP In this way it is theoretically possible to recover one "energy-rich" bond from the breakdown of lecithin

It is unlikely that phospholipase C or phospholipase D are important in the degradation of lecithin in animal tissues However, phospholipase C

plays an important role in the breakdown of lecithin in certain microorganisms and phospholipase D is active in many plants.

C PHOSPHATIDYL ETHANOLAMINE

In animal tissues phosphatidyl ethanolamine (III) is broken down by a series of reactions analogous in every way to those responsible for the degradation of lecithin. Despite the report of Chargaff and Cohen (*133*) that the phospholipase A of snake venom is inactive toward cephalin, Dunn (*180*), Fairbairn (*134*), and Long and Penny (*148*) reported good activity. Phospholipase A removes the molecule of fatty acid esterified in the α' position of phosphatidyl ethanolamine by a reaction analogous to reaction (18), with the formation of lysophosphatidyl ethanolamine.

Fairbairn (*152*) showed that lysophosphatidyl ethanolamine is degraded further, to give fatty acid and GPE [cf reaction (19)], by a phospholipase B present in *Penicillium notatum*. Subsequently Dawson (*156*) showed that a similar enzyme is present in rat and sheep liver. Dawson (*159a*) also reported that liver contains a lipid fraction that enables the phospholipase B of *P. notatum* to attack phosphatidyl ethanolamine with the production of GPE and free fatty acids (cf. similar observations described above for lecithin)

Phosphatidyl ethanolamine is probably broken down by the successive action of phospholipase A and phospholipase B in animal tissues, for Schmidt *et al* (*181*) showed that the intestinal mucosa of the rat contains an enzyme capable of liberating GPE from phosphatidyl ethanolamine and many workers have demonstrated the presence of GPE in a wide variety of animal tissues (*50, 182–184*). By studying the time course of the labeling of GPE and phosphatidyl ethanolamine in rat liver, Dawson (*50*) concluded that the GPE is a degradation product of phosphatidyl ethanolamine and not a precursor, just as GPC is a degradation product of lecithin.

The GPE formed from the breakdown of phosphatidyl ethanolamine may be hydrolyzed further to GP and ethanolamine [cf. reaction (22)] by the diesterase present in liver (*175*). In passing it should be noted that many tissues contain a phosphomonoesterase capable of hydrolyzing PE (*177, 179*). This enzyme probably plays little part in the degradation of phosphatidyl ethanolamine in animal tissues.

Bacterial phospholipase C is inactive toward phosphatidyl ethanolamine (*160, 161*), but the plant phospholipase D removes ethanolamine, with the formation of phosphatidic acid (*125, 170, 172a*). There is no evidence that either of these enzymes play a role in the metabolism of phosphatidyl ethanolamine in animal tissue.

D. Phosphatidyl Serine

It is probable that phosphatidyl serine (IV) is broken down in animal tissues by a series of reactions similar to those described for lecithin and phosphatidyl ethanolamine, but the evidence is not so complete

Long and Penny (*148*) showed that the venom phospholipase A removes a molecule of fatty acid from phosphatidyl serine to form lysophosphatidyl serine [cf reaction (18)] and Fairbairn (*152*) reported that phospholipase B of *Penicillium notatum* removes a further molecule of fatty acid from lysophosphatidyl serine to form GPS [cf reaction (19)]. Recently Schmidt *et al* (*181*) reported that the intestinal mucosa of the rat contains a system of enzymes capable of liberating GPS from phosphatidyl serine, indicating that the hydrolysis of phosphatidyl serine by phospholipase A and phospholipase B probably occurs in animal tissues

Bacterial phospholipase C does not remove phosphorylserine from phosphatidyl serine (*160, 161*) but, as found for phosphatidyl ethanolamine, plant phospholipase D is active (*125*) There is no evidence that phosphatidyl serine is degraded by enzymes such as these in animal tissues

E Plasmalogen

Long and Penny (*148*) showed that phospholipase A can remove a molecule of fatty acid from ethanolamine plasmalogen (VII or VIII). Rapport and Franzl (*185*) reported similar findings for choline plasmalogen, although apparently the plasmologen is hydrolyzed more slowly than the analogous diester lecithin (*150b*) From the experiments of Kiyasu (*95*) referred to earlier it might also be inferred that phospholipase C is active in removing PC from choline plasmalogen

F Sphingomyelin

Little is known of the enzymic degradation of sphingomyelin (IX) There are reports in the literature of an uncharacterized "sphingomyelinase" in various animal tissues (*186, 187*) Thannhauser and Reichel (*188*) and Fujino (*189*) presented evidence that liver and pancreas contain an enzyme that splits off PC from sphingomyelin, with the formation of a fatty acid derivative of sphingosine (ceramide) (X), probably

sphingosine (ceramide)



The finding that bacterial phospholipase C can remove PC from sphingomyelin raises the question as to whether some of the reports of the breakdown of sphingomyelin in tissue preparations might not be the result of bacterial contamination.

Earlier Magistris (*190*) reported the presence of an enzyme in bee venom that liberates lignoceric acid from sphingomyelin with the formation of "lysosphingomyelin" (sphingosylphosphorylcholine) However, the identity of this enzyme has been questioned [see (4)]. Subsequently Fairbairn (*134*) reported that venom phospholipase A does not hydrolyze sphingomyelin. The presence of sphingosylphosphorylcholine in animal tissues has been reported (*191*, *192*), but Dawson (*193*) could not confirm this finding. At the present time there is no good evidence that phospholipase A, or any other enzyme that removes a fatty acid from sphingomyelin, occurs in nature.

G Inositol Phosphatide

Sperry (*194*) and Tyrrell (*195*) reported that when brain homogenates are incubated some of the lipid phosphorus is lost Johnson *et al* (*196*) showed that under similar conditions lipid phosphorus is lost from slices of cat brain and from fragments of peripheral nerve Most of the lipid phosphorus that is lost is derived from the "cephalin" fraction (*195*, *196*) Subsequent work by Sloane-Stanley (*197*) and Rodnight (*198*) indicates that this phosphorus is derived from brain diphosphoinositide. Prominent among the hydrolysis products are inorganic phosphate and inositol monophosphate The enzyme system responsible for the hydrolysis is widely distributed in mammalian tissues (*198*)

Few studies on the degradation of isolated phosphatidyl inositol (XII) have been published. Long and Penny (*148*) and Hanahan and Olley (*107*) reported that inositol glycerophosphatide is not acted upon by venom phospholipase A. However, Hawthorne and Kemp (*199*) recently described the preferential enzymic destruction of phosphatidyl inositol by extracts of rat liver and heart Water-soluble organic phosphate and some inorganic P are among the end products of the hydrolysis

III. Turnover of Phosphatides

The evidence from isotope experiments is that in most animal tissues the phosphatides are in a dynamic state, i e , they are continually being built up and broken down An interesting exception is the myelin sheath that surrounds the axon of nerve cells (*200*).

In the preceding sections an attempt has been made to summarize existing knowledge of these processes. It seems probable that the reactions leading to the formation of phosphatides are quite different from those

ing to their destruction Thus in animal tissues there is a continuous cycle of anabolic and catabolic reactions, as suggested by Dawson (43). For lecithin, this cycle of anabolic and catabolic reactions is summarized in Fig 6 The figure emphasizes that in the anabolic reactions,

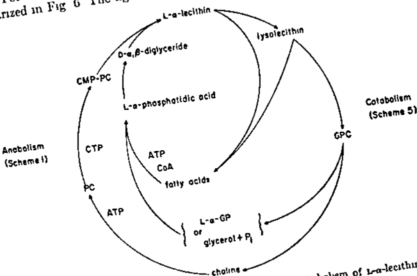


FIG 6 Schematic representation of anabolism and catabolism of L-α-lecithin in animal tissues

previously presented in Scheme 1, PC and CMP-PC are intermediates, whereas in catabolic reactions, previously presented in Scheme 5, the principal choline-containing intermediate is GPC

It is regretted that of necessity much of this chapter has been devoted to lecithin, and to its metabolism in animal tissues It would be highly desirable to have comparable information for other phosphatides and for tissues other than those of animal origin

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Metabolism of Sterols*

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* The following abbreviations are used in this chapter DPN and TPN, di- and triphosphopyridine nucleotide, DPN⁺, TPN⁺, DPNH, and TPNH: the oxidized

I. Introduction

A. Scope of Chapter

This chapter summarizes the current knowledge concerning the metabolism of sterols. The biogenesis of cholesterol, of the plant sterols, and of the bile acids is reviewed We shall also discuss briefly the homeostatic (or feedback) control mechanism which serves to maintain the blood cholesterol at a relatively constant level The literature on these subjects is so vast that no attempt is made to give a complete review of all the papers Instead, greater emphasis will be placed on the more recent developments and, for the older literature, the reader is referred to the numerous reviews (*1–20*) listed at the beginning of the bibliography The biogenesis and the metabolism of the steroid hormones will not be included in this chapter since these form the subject of discussion of another chapter

B. Brief Historical Development

The modern biochemistry of the sterols may be considered to begin with the classic experiment of Rittenberg and Schonheimer (*21*) who concluded, by the use of D_2O, that cholesterol is synthesized from small molecules Shortly afterward, it was shown that acetate is a major precursor of both ergosterol (*22*) and cholesterol (*23*). The degradation of the side chain of cholesterol biosynthesized from 1-C^{14}- or 2-C^{14}-acetate revealed a similar labeling pattern (*24*) to that in rubber (*25*) and thus led Bloch and co-workers to suggest the close relationship between sterols and terpenes and to revive the hypothesis that squalene is an intermediate in the biogenesis of cholesterol (*26–28*). This was finally proven correct in 1953 by Langdon and Bloch, who isolated labeled squalene from rats given labeled acetate and, using this squalene, demonstrated the *in vivo* conversion of squalene to cholesterol (*29, 30*) At about the same time, Bucher (*31, 32*) made an important contribution when she succeeded in preparing a cell-free homogenate of liver which is capable of converting acetate to cholesterol, thus opening the problem of cholesterol biogenesis to standard enzymological approach. Soon after this, another milestone was erected by the work of Ruzicka and co-

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and reduced forms of above, ATP, ADP, and AMP· adenosine tri-, di-, and monophosphate, CoA-SH coenzyme A, MVA mevalonic acid, HMG· β-hydroxy-β- [illegible] acid, [illegible] the [illegible] the

phosphate group is represented by P

workers who identified lanosterol as having the same steroid nucleus as cholesterol (*33*). Assuming lanosterol to be a precursor of cholesterol, Woodward and Bloch (*34*) proposed and obtained experimental evidence for a revised squalene hypothesis. This hypothesis has since been firmly established by the following developments. The complete chemical degradation of the sterol ring system (*35–39*) and of squalene (*40*), achieved mainly through the work of Cornforth and Popják and their co-workers, revealed that the labeling pattern of the two carbons of acetate in these two compounds agreed with the prediction of the Woodward-Bloch hypothesis. In 1956, Clayton and Bloch succeeded in demonstrating the *in vivo* and *in vitro* synthesis of lanosterol (*41–42*). The conditions under which lanosterol is formed indicated that it must be identical to one of the "high counting companions" previously reported by Schwenk *et al.* to be precursors of cholesterol (*43–46*). A detailed mechanism of the cyclization of squalene to lanosterol and to the various cyclic triterpenes was proposed by Ruzicka and co-workers in 1953 and 1955 (*47–48*) and received strong experimental support from the Bloch school (*49–52*). The Bloch school also showed that the further conversion of lanosterol to cholesterol involves oxidative demethylation (*53*). Among the plant sterols, several groups have studied the formation of ergosterol from acetate and showed that, like cholesterol, all the carbons were derived from acetate except the extra methyl group in the side chain (*22, 39, 54, 55*). This latter carbon in the sterols was shown by several laboratories to derive from the one carbon pool, most likely S-adenosyl methionine (*56–62*).

With respect to the intermediates between acetate and squalene, little was known up to 1956 despite the efforts of several laboratories to identify the "biological isoprene unit" [see reference (*1*)]. In 1956, the key discovery was made by Tavormina, Gibbs, and Huff that mevalonic acid (MVA, β-hydroxy-β-methyl-δ-valerolactone), an acid isolated as a growth factor for *Lactobacillus casei* (*63*) was an efficient precursor of cholesterol (*64, 65*). This was confirmed by many laboratories and it was further shown that phosphorylated derivatives are involved in the transformation of MVA to squalene (*66–77*). The detailed mechanism of this transformation will probably be elucidated in the near future. The efficient utilization of MVA to form rubber and various terpenoid derivatives has also been reported (*20, 78–81*).

The above discussion renders clear the close relationship between the sterols and the terpenes and permits the reasonable assumption that all these compounds are derived from a common "biological isoprene unit." The problem of cholesterol biogenesis may thus be logically divided into several phases. (1) The identity and the formation of the "biological

isoprene unit," (2) the condensation of these units to form the acyclic triterpene squalene, (3) the cyclization of squalene to lanosterol, i e, the formation of the sterol ring system, and (4) the transformation of lanosterol to cholesterol The following sections will discuss these phases in this order, to be followed by brief discussions on the control of cholesterol biosynthesis and the formation of bile acid and of fecal sterols

II. The Identity of the "Biological Isoprene Unit"

A. β-Hydroxy-β-Methylglutaric Acid and Other Branched Chain Acids

The first experimental evidence that the sterols may be related to the terpenes came from the study of the distribution of the carbon atoms of acetate in the side chain of cholesterol (*24*). Using acetate labeled with C^{14}, it was observed that the distribution of the methyl carbon and the carboxyl carbon in the side chain of cholesterol is similar to that suggested for rubber, a known polyisoprenoid (Fig. 1) The identity of the

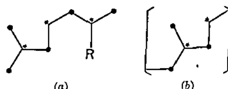


FIG 1 Distribution of the methyl carbon and the carboxyl carbon of acetate in the side chain of cholesterol (a) and in rubber (b) methyl carbon, dots, carboxyl carbon, stars

"biological isoprene unit" thus became the crucial point in the biogenesis of cholesterol as well as in the biogenesis of the terpenes and of rubber Various 5-carbon and 6-carbon branched chain acids were tested for this role, but none was found to be as effective as acetate as a precursor of cholesterol when assayed with liver homogenates (*82*) *In vivo*, however, some of the acids tested were found to be more efficiently converted to cholesterol than was acetate (*82, 83*) Furthermore, the degradation of the cholesterol formed *in vivo* from dimethylacrylic acid indicated that this acid could be converted into cholesterol without prior breakdown to acetate Thus, using 3-C^{14}-labeled dimethylacrylic acid, it was found that the cholesterol formed from this labeled precursor had no C^{14} in the C-23 of the side chain (*84*) This clearly showed that dimethylacrylic acid was not degraded to acetate and then subsequently incorporated into the sterols and suggested that there is a precursor of cholesterol which is structurally similar to dimethylacrylic acid Such 5-carbon units may

arise biologically from the condensation of three molecules of acetate with decarboxylation Such a scheme was first proposed by Bonner and Arreguin (*25*) and would give the observed distribution of the carboxyl and the methyl carbon atoms of acetate in the "isoprene unit" (Fig 2).

There was, however, other evidence suggesting that, although these 5-carbon acids could be incorporated into cholesterol without breakdown

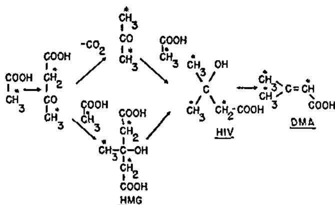


FIG 2 Hypothetical scheme of conversion of acetate to the "biological isoprene unit"

to acetic acid, they were nevertheless not directly related to the "biological isoprene unit" Thus, it was shown that the 5-carbon acids and CO_2 exerted a mutually stimulatory effect on each other's incorporation into cholesterol (*84, 85*) These results suggested that a 5-carbon acid was not the direct precursor of cholesterol but that it must be carboxylated to give a 6-carbon acid which was then converted to the sterols. Such a carboxylation reaction had been demonstrated by Coon and co-workers in the course of their systematic study of leucine breakdown which follows the sequence of reactions given in Fig 3 (*86–88*) It should be noted that the carboxylation of the CoA derivative of the 5-carbon acid led to the formation of the same acid postulated in the condensation of activated acetate, namely, HMG-SCoA. Assuming that HMG-SCoA is an intermediate in the biogenesis of cholesterol, these reactions would then explain the stimulatory effect of CO_2 on the incorporation of the 5-carbon acids into cholesterol Combination of the CO_2 fixation enzyme and the cleavage enzyme hydrolyzing HMG-SCoA to Ac-SCoA and acetoacetate would lead to the incorporation of $C^{14}O_2$ into the carboxyl group of

acetoacetate. Since previous work has already shown that acetoacet[...] can be converted to cholesterol (*89–91*), but only after prior conversi[...] to acetate (*92*), the labeling pattern of the cholesterol arising from C^{14}[...] should be the same as with carboxyl-labeled acetate This agrees w[...] and explains the labeling pattern of cholesterol derived from $C^{14}O_2$ a[...] nonlabeled DMA (*84, 85*).

More recently, Rudney and co-workers have demonstrated the f[...]mation of HMG-SCoA from acetoacetyl-SCoA and acetyl-SCoA (*15*, [...] and showed that the product is identical to the HMG-SCoA formed fr[...]

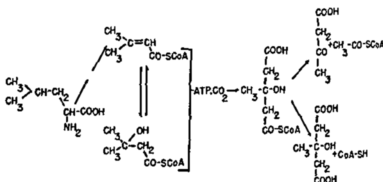


Fig 3 Formation of HMG-SCoA in leucine breakdown.

the C_5 acids by CO_2 fixation (*94*) In the past year, they have furth[...] succeeded in demonstrating the enzymic reduction of HMG-SCoA [...] MVA (*15, 95*), which has been established as a precursor of cholester[...] and will be discussed in detail in the following paragraphs. Independentl[...] similar results were obtained in Lynen's laboratory (*16*). It is thus firm[...] established that HMG-SCoA is a key intermediate in the synthesis [...] cholesterol. Not only acetate, but also the various 5-carbon acids men[...]tioned above are all converted to HMG-SCoA before being utilized f[...] the synthesis of cholesterol A detailed discussion of the metabol[...] interrelationship among these acids and their role in the formation [...] ketone bodies is given elsewhere in this book, and so will not be include[...] in this chapter Instead, a diagram is presented (Fig 4) which illustrat[...] the most likely interrelationship between these acids as demonstrate[...] in the above mentioned work and in some more recent results fro[...] Coon's laboratory (*96*) [reaction (a)] and from Lynen *et al.* (*16, 97–9*[...] [reactions (a) and (b)] Based on this reaction scheme, and on the reac[...]tion sequence between MVA and squalene to be discussed in the followin[...] sections, one may predict the distribution of C^{14} in the side chain [...]

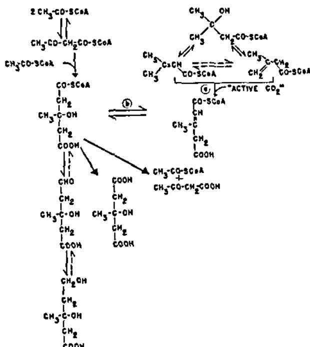


FIG 4 Metabolism of HMG-SCoA Two routes of synthesis and three of breakdown

cholesterol from various C^{14}-labeled precursors This is shown in Fig 5 and agrees well with the experimental finding [1]

B The Discovery of Mevalonic Acid as Precursor of Cholesterol

In 1956, the research group at the Merck, Sharp and Dohme Laboratories identified a growth factor for a *Lactobacillus* as β-hydroxy-β-methyl-δ-valerolactone and named it mevalonic acid (*100*) Noting the similarity between this acid and HMG, they tested it as a precursor for cholesterol synthesis. Using a synthetic racemic mixture, 43% of the acid was incorporated into cholesterol (*64*) It was subsequently shown by several groups of workers that MVA is converted to the triterpene squalene and thence to cholesterol without breakdown to smaller units (*66–68*) Degradation of the squalene synthesized from 2-C^{14}-MVA

[1] There is one compound, 1-C^{14}-DMA, which does not agree with this scheme This was discussed in reference (*1*) Since no explanation can be offered now for this puzzle, the interested reader is referred to the above mentioned article.

FIG 5 Labeling pattern of cholesterol side chain from various labeled precursors

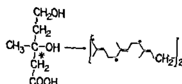


FIG. 6 Labeling pattern of squalene derived from 2-C^{14}-MVA

revealed the expected labeling pattern (Fig 6) It is thus clearly established that MVA is an intermediate in the biogenesis of squalene.

C THE OXIDATION-REDUCTION LEVEL OF THE CONDENSING UNIT

Although MVA is an intermediate in squalene biogenesis, it is unlikely, due to the lack of reacting groups in the molecule, that two molecules of MVA would undergo condensation. Using chemical and biochemical

analogies, one would expect the molecule of MVA to undergo one of the following types of reactions to give a derivative that is sufficiently reactive to undergo condensation reactions. (1) Oxidation of the C-5 of mevalonic acid to an aldehyde, (2) oxidation of the C-5 to a carboxyl group or its derivative, and (3) formation of double bonds between carbons 2 and 3 and between 4 and 5. In the first two cases, the oxidized MVA would undergo condensation in manners similar to known biochemical reactions In the latter case, one has only chemical analogy to cite as model reactions These different mechanisms were examined with the use of D- and T-labeled MVA and with D_2O as the reaction medium (*69, 101–103*). The results with heavy hydrogen-labeled MVA in position five shows that at least ¾ of the hydrogens of the C-5 of MVA was retained in the squalence (*69, 102*) Since an oxidation of the alcohol *group to an aldehyde or to an acid would result in the loss of half or all* of the hydrogens on C-5, both these possibilities were eliminated by the results obtained with D- and T-labeled substrate

Further experiments with D_2O as the reaction medium confirmed and amplified these results (*101–103*) When MVA was converted to squalene in a medium of D_2O, there was incorporated into squalene about four atoms of D [2] If the reaction were carried out in a medium of H_2O but with CH_3CD_2OH-DPN-alcohol dehydrogenase as the source of reductant,[3] the squalene isolated contained less than half an atom of D From these two sets of experiments, the total hydrogen uptake from the environment during the transformation of MVA to squalene was determined to be four

If one examines the structural changes in the formation of squalene from MVA, one can predict a minimum number of hydrogens that must be incorporated into squalene from the medium Thus, at the two ends of the squalene molecule, one sees that two of the $—CH_2COOH$ groups of the MVA have been transformed to CH_3 groups Such changes must lead *to the formation of two new C—H bonds with the uptake of two atoms* of D if the reaction was carried out in a medium of D_2O Also, the formation of squalene from MVA is a reductive process, with the uptake of one mole of hydrogen per each mole of squalene formed If the reaction was carried out in D_2O with $CH_3—CD_2OH$ as the only source of reductant, the reduction must be accompanied by an uptake of 2 D per each molecule of squalene formed There is thus a minimum uptake of four

[2] In this discussion of work using D as tracer, only D bound to C in a stable *manner is considered Any D on the hydroxyl groups exchanges readily with proton* in the solvent and is removed during the isolation of the compounds

[3] The over-all conversion of MVA to squalene is a reductive process This point will be discussed later

atoms of D from the medium into each molecule of squalene.[4] The experimental value observed was four. Therefore, there could not be any other steps involved in the transformation of MVA to squalene which would necessitate the incorporation of D into the molecule. It also follows that the carbon atoms 2 and 5 of MVA, which are present as $-CH_2-$ both in MVA and squalene, must have retained both H atoms throughout

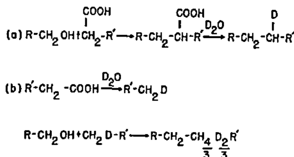


FIG 7 Condensation mechanism excluded by the D data

the transformation. From these considerations, several types of reactions can be excluded in the biogenesis of squalene from MVA:

(1) Oxidation of the alcohol group at C-5 to an aldehyde or to an acyl-derivative. Such a mechanism would remove one or both of the H on the carbon 5 of MVA Since these carbon atoms remain as CH_2 groups in the squalene formed, oxidation prior to condensation must result in the uptake of hydrogen from the medium This is therefore ruled out by the experimental result

(2) Decarboxylation following condensation: If the condensation was to take place with the carboxyl groups still attached, the subsequent decarboxylation must lead to the incorporation of D from the medium (Fig 7,a)

(3) Decarboxylation prior to condensation but with the formation of a CH_3 group Such a mechanism would lead to the introduction of one atom of D per each methyl group formed In the subsequent transformations, two of these methyl groups are converted to the terminal methyl groups of squalene, and four are converted to the methylene groups in squalene The two methyl groups at the end would contain two D as

[4] There is the possibility that the two D incorporated in the reductive process are the same two that we have postulated for the two ends of squalene. This possibility is discarded in this discussion for two reasons First, it does not invalidate any of the conclusions to be drawn, provided that minor nonessential modifications be made Second, as will be discussed later, this has been shown to be not the case

discussed previously, but the four methylene groups would now also contain D to the extent of 4 × ⅔ atoms of D per molecule of squalene. This again is at variance with the experimental result (Fig 7,b).

With these three possibilities eliminated, it becomes possible to consider some of the features that the condensing isoprenoid intermediate must possess In the first place, the interaction must be between two methylene groups. The simplest scheme that one can write is to assume that the interaction is between two terminal methylene groups If the

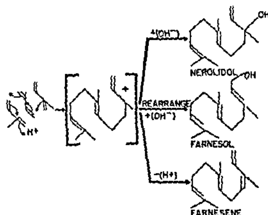
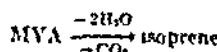


FIG 8 Hypothetical scheme of concerted condensation of three isoprene molecules

condensing unit has two terminal methylene groups, the only possible structure is that of isoprene itself. Theoretically, one can write the condensation of three isoprene molecules to give all three naturally occurring noncyclic sesquiterpenes (Fig. 8). Such a scheme has the advantages (1) that all the double bonds end up in the right position, (2) that one can arrive at any of the three natural acyclic sesquiterpenes, (3) that it is the simplest mechanism and (4) that isoprene can be derived, on paper at least, from MVA very readily



$$\text{MVA} \xrightarrow[-CO_2]{-2H_2O} \text{isoprene}$$

There are, however, also severe drawbacks in this mechanism, since it is hard to visualize free isoprene molecules being formed in biological systems which are at a higher temperature than the boiling point of isoprene. And indeed, all attempts to demonstrate the formation of

isoprene or the conversion of isoprene to squalene have been unsuccessful[5] (*17, 104*) and it is most unlikely that isoprene itself is involved in the biogenesis of the steroids However, as will be shown later, the most likely scheme of condensation between the isoprenoid units is really very similar to this mechanism and may be considered as a variation of it. It is for this purpose that the scheme for the condensation of three isoprene molecules is presented here

D Phosphorylated Intermediates

When the requirement for ATP was discovered, there were essentially two possible functions that could be postulated for it First, ATP may be required to form an active acyl group so that condensation may occur between the adjacent CH_2 group and an aldehyde or an acyl-CoA group of another molecule of the condensing unit This was rendered unlikely when it was found that the condensing unit did not have aldehyde or carboxyl groups, and that during the condensation, there was no loss of hydrogen from the CH_2 groups that are derived from the C-2 of MVA (*69, 101–103*). This then leaves the other alternative, that ATP is required because phosphate esters are involved in the transformation of MVA to squalene

This second hypothesis was borne out by the discovery of several phosphorylated derivatives in the conversion of MVA to squalene Recent results indicate that the reaction sequence shown in Fig 9 probably takes place.

The first reaction was demonstrated to occur in yeast autolyzate by Tchen in 1957 (*70*) The product was identified as a phosphate ester of MVA Later, it was proved both indirectly by the use of labeled compounds (*75*) and directly by chemical synthesis [Lynen and co-workers (*16, 73*)] that the phosphate is attached to the 5-hydroxyl group of MVA The same intermediate was also shown by Popják and co-workers to be formed by liver homogenate (*74*)

The formation of the pyrophosphate of MVA was also demonstrated by the use of yeast extract (*75, 77*) Its structure was deduced from the following pieces of evidence (1) It contains two moles of P per each mole of MVA (2) It still retains the carboxyl group of MVA (3) One of the two phosphates, the one derived from ATP in reaction (b), is readily hydrolyzed off by acid (4) In its subsequent conversion to the pyrophosphate of Δ^3-isopentenol, both phosphate groups are retained, one in an

[5] Isoprene labeled with T was incubated in a closed system with yeast or yeast autolyzate, with or without the addition of MVA The squalene isolated and purified was found to contain no T (*104*). Negative results were also reported by Popják and Cornforth and their co-workers (*17*).

acid-labile linkage. (5) Its acid hydrolysis gives a product identical to P-MVA. From this evidence, only one reasonable structure can be assigned to this compound, namely 5-pyrophosphomevalonic acid. A similar diphosphorylated derivative of MVA was also reported by Popják and co-workers (74).

The third reaction was postulated to explain the ATP requirement in the decarboxylation of PP-MVA and the formation of Δ^3-isopentenol pyrophosphate. Although ATP is required, no P from ATP is incorporated

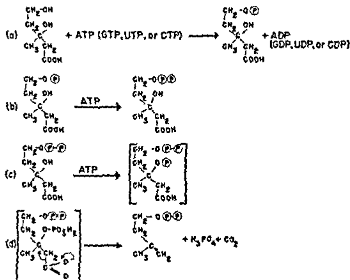


FIG. 9. Phosphorylated derivatives of MVA

into Δ^3-isopentenol pyrophosphate. A 3-phosphorylated derivative was postulated to explain this ATP requirement. The conversion of the hydroxyl group to a phosphate ester is expected to facilitate the cleavage of the C—O bond and thus aid the elimination of the 3-hydroxyl (or phosphate) group and the carboxyl group. As was pointed out earlier (Section II,C), the removal of the carboxyl group must be accompanied by the elimination of the 3-OH group, and such a concerted process is illustrated in reaction (d) in Fig. 9. The product of this reaction was [illegible]

II,C, it must contain the C-2 of MVA as a terminal methylene group and

cannot have a hydroxyl group (or phosphate) on C-3. (The presence of a methylene group, a methyl group, a —CH_2—CH_2—O—(P—P) and a hydroxyl group would make C-3 a pentavalent carbon) (3) Enzymic hydrolysis of the two phosphate groups (by snake venom) gives rise to Δ^3-isopentenol More recently, Lynen *et al* *have synthesized chemically* Δ^3-isopentenol pyrophosphate and showed that it is identical to the *enzymic product (76)*

Δ^3-Isopentenol pyrophosphate can be converted to C_{15} and C_{30} units in the absence of ATP *(73, 75, 76)* *With this discovery, the long search* for the "biological isoprene unit" has finally reached its end. Discounting possible transient unstable enzyme-bound derivatives of it, Δ^3-isopentenol pyrophosphate may be considered as the "biological isoprene unit"

III. The Condensation of Δ^3-Isopentenol Pyrophosphate and Farnesyl Pyrophosphate

A Formation of C_{15} Unit and Isomerization of C_5 Unit

As was mentioned in the preceding paragraph, Δ^3-isopentenol pyrophosphate can be converted to C_{15} and C_{30} units in the absence of ATP *Lynen and co-workers succeeded in demonstrating its enzymic conversion* to farnesyl pyrophosphate (76) The carbon skeleton of this compound was identified by the successive action of phosphatases (snake venom) and alcohol dehydrogenase (and DPN^+) which resulted in the formation of farnesal The latter was identified as its dinitrophenylhydrazone The formation of farnesyl pyrophosphate suggests the scheme of condensation of the C_5 units as shown in Fig 10 [6] The condensation of three Δ^3-isopentenol pyrophosphate *would lead to the formation not of* farnesyl pyrophosphate, but of an isomer of it It is thus evident that, at one stage or another, an isomerization must have taken place; this is indicated by the dotted arrows Recent work by Lynen and his co-workers have shown that yeast extract contains an enzyme catalyzing the isomerization of Δ^3-isopentenol pyrophosphate Whether the alternative pathways of condensation also occur remains to be determined It might be mentioned that although *farnesol pyrophosphate* has been isolated and identified, very little C_{10} (geraniol or its isomer) pyrophosphate has been encountered *It is quite possible that this intermediate is* enzyme bound

co-workers (76)

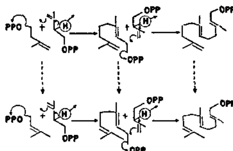


FIG 10 Stepwise condensation of Δ^3-isopentenol pyrophosphate

and not liberated into solution [7] If such is the case, then it may be reasonable to assume that the isomerization occurs either at the C_5 or at the C_{15} stage, most likely only at the C_5 stage

B Condensation of Farnesyl Pyrophosphate

The coupling of two farnesyl pyrophosphate to give squalene is a reductive process In the early work on the conversion of MVA to squalene, it was found that a reductant (DPNH or TPNH) was required (*16*, *69*). Subsequent work has shown that MVA can be converted to farnesyl pyrophosphate in the absence of DPN; and, if one examines the structure of these compounds, it can be readily seen that the formation of farnesyl pyrophosphate from MVA involves no oxidation-reduction while the conversion of two moles of farnesyl pyrophosphate to squalene must be accompanied by an uptake of one mole of hydrogen

The mechanism of this coupling remains unknown, although some intelligent speculation can be made A theory was proposed earlier that this coupling might be achieved by a reductive coupling of two moles of farnesene (*101*) This has now been shown to be incorrect by the same workers (*103*) Squalene was isolated from two types of experiments. one using MVA labeled with T on C-5, and another using MVA in a medium of D_2O (and CH_3—CD_2OD as reductant). The squalene was degraded by ozonolysis and the center four carbons isolated as succinic acid. It was found that in the case of T-labeled MVA, the succinic acid isolated contained only half the theoretical amount of T. In the case of MVA in D_2O, the succinic acid contained two atoms of D This indicated that (1) during the conversion to squalene, the two farnesyl pyrophosphate molecules lose two of the hydrogen attached to the terminal —CH_2—OPP

[7] More and more examples of such enzyme-bound intermediates are being found Thus, in the activation of acids by the formation of acyl-AMP derivatives, these compounds are as a general rule firmly bound to the enzymes

group; (2) two atoms of hydrogen are added to these carbons, probably during the reduction; and (3) all the other carbons in farnesyl pyrophosphate retain all their hydrogen atoms throughout this reductive coupling process [8] Based on these conclusions, there are only a limited number of possible mechanisms. All of these are similar to and may thus be represented by the sequence of reactions in Fig 11

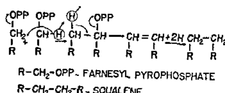
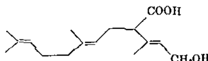


FIG 11 Coupling of farnesyl pyrophosphate

C. "POLYMERS" OF MVA

A family of acidic derivatives of MVA have been reported by Popják and co-workers (*17, 74*) [9] MVA was incubated with liver preparation and ATP in the absence of DPN After acidification of the reaction mixture and extraction with organic solvent, a group of nonphosphorylated acidic derivatives of MVA was obtained Vapor phase chromatography revealed the heterogeneity of this extract, showing the presence of many components The major components behaved (in vapor phase chromatography) like C_{11}, C_{12}, C_{15}, C_{16}, or C_{17} acids. Minor compounds were also observed which could have possibly 21, 22 or perhaps even more carbon atoms These materials, contrary to the C_{10}, C_{15}, and C_{30} compounds discussed in the preceding sections, still contained the carboxyl group of MVA These compounds have not been encountered in the work with yeast extracts, but are the main product of reaction when MVA is incubated with liver preparation in the absence of DPN, and have also been observed by Ogilvie and Langdon who assign the structure



[8] As discussed earlier, there is a total incorporation of 4 D per molecule of squalene The other two D has been proven to be located at the two ends of squalene. By ozonolysis the two Me_2C = groups at the two ends of squalene were obtained as acetone and found to contain D as expected No D was found in levulinic acid, it can be concluded, therefore, that all the other carbons must have retained all the hydrogens attached to them

[9] This observation was discussed in considerable detail in the "Discussion" following reference (*17*)

to the main component (*105a,b,c*). When reincubated with liver homogenate and all the cofactors, these acidic compounds are not converted to squalene or to cholesterol The significance of these compounds thus remains unknown

IV. Cyclization of Squalene

A THE TWO SQUALENE HYPOTHESES

In 1934, Sir Robert Robinson proposed that squalene can be cyclized to a tetracyclic derivative which, with minor structural changes, can be converted to cholesterol (*28*). This hypothesis became more than a theoretical possibility after the demonstration by Langdon and Bloch

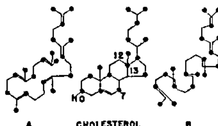


FIG 12 Labeling pattern of the carbon atoms of acetate in cholesterol and in squalene The squalene molecule on the left is folded according to Robinson, and the one on the right according to Woodward and Bloch.

in 1953 that squalene is synthesized from acetate in rat liver and that the biosynthesized squalene can be further converted to cholesterol (*29, 30*). At about the same time that the biochemists were engaged in the study of the role of squalene in sterol biogenesis, several organic laboratories were engaged in the elucidation of the structure of the cyclic triterpenes. Among these, one compound, lanosterol, proved of particular significance. As a result of these elegant studies lanosterol was shown to be a triterpene with the steroid nucleus (*33*), and thus pointed to the possibility that lanosterol may be an intermediate in the biogenesis of cholesterol. Woodward and Bloch reexamined the squalene hypothesis in the light of the structure of lanosterol and proposed a new scheme of squalene cyclization (*34*) This scheme can be experimentally differentiated from that of Robinson in the following manner. If methyl-labeled acetate were used in the biosynthesis of cholesterol, the labeling pattern of carbon atoms 7, 8, 12, and 13 of cholesterol would be different, depending upon the mechanism of cyclization of squalene, as illustrated in Fig 12. The carbon atom 13 would be labeled according to the Woodward-Bloch mechanism and unlabeled by the Robinson mechanism. Degradation of cholesterol

biosynthesized from methyl-labeled acetate yielding carbon atoms 13 and 19 as acetate proved that carbon atom 13 in cholesterol is derived from the methyl carbon of acetate, in agreement with the Woodward-Bloch mechanism of squalene cyclization. Independently, the same conclusion was reported by Dauben *et al.* (*105d*) based on similar experimental evidence. When the degradation of the steroid nucleus was completed, the labeling pattern of carbons 7 and 12 of cholesterol and ergosterol synthesized from labeled acetates was shown to be also in accord with the prediction of the Woodward-Bloch mechanism (*35–39*) In 1956, the scheme was further confirmed when labeled lanosterol was formed from acetate and was shown to be converted to cholesterol (*41, 106*).

B THEORETICAL CONSIDERATIONS OF SQUALENE CYCLIZATION

With both squalene and lanosterol established as intermediates in the biogenesis of cholesterol, one can approach the problem of the formation of the nucleus of the sterols In 1953, Ruzicka and co-workers proposed that this apparently complex transformation could be achieved by a concerted cyclization of squalene followed by hydride and methyl shifts (*47*) Later, these workers extended the concerted mechanism to all the cyclic triterpenes and postulated the stereochemical changes that must accompany the formation of each of these compounds (*48*). It is unfortunately impossible to condense this elegant work into one or two pages for inclusion in this chapter We shall, therefore, only consider here the first step in the cyclization of squalene to the two stereoisomeric triterpenes tirucallol and lanosterol (Fig. 13)

The initiation of this cyclization is represented by an electrophilic attack of a hypothetical oxidant OH^+. This should not be taken literally to imply that OH^+ ions exist in biological systems and participate in biological oxidations As will be shown in the following section, the oxidant is "activated molecular oxygen" whose identity is still unknown

The squalene molecule is postulated to be folded in definite geometrical configuration on the enzyme surface such that the π-electrons in the double bonds are coplanar and close together. Of the many possible ways of "folding" the squalene molecule, two are shown in Fig 13 Concerted electron shifts following the electrophilic attack by OH^+ leads to ring closure and the formation of two transient intermediary carbonium ions I and II With the exception of lanosterol, the carbon skeleton of all the tetra- and penta-cyclic triterpenes, whose structure was identified by 1958, can be derived from carbonium ion I by a series of concerted hydride and methyl shifts However, in order to form lanosterol from squalene in a fully concerted manner, one must postulate a different intermediary carbonium ion II. The "folding" of squalene to give rise to these two

carbonium ions is shown in Fig 13 It should be mentioned here that more recent work have suggested that cyclization of squalene takes place in nature with a third "folding" (conformational isomer) of squalene (*20*).

This theoretical treatment by Ruzicka and associates on the mechanism of squalene cyclization not only explains satisfactorily the formation of these complex and yet similar structures, but also raises an interesting

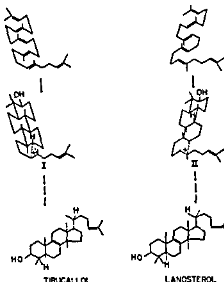


FIG 13 Two intermediary carbonium ions (I and II) formed by the oxidative cyclization of squalene I has all three six-membered rings in the chair form whereas II has the B ring in the boat form By a parallel series of rearrangements, I and II give rise to tirucallol and lanosterol respectively I can also rearrange to give the other tetracyclic and pentacyclic triterpenes

point in comparative biochemistry Assuming that the steric course of the cyclization is governed by enzymes, and that there are many different cyclizing enzymes leading to the formation of the many cyclic triterpenes, one would conclude that the structure of these enzymes would be very similar to each other except for the one which forms lanosterol An examination of the known triterpenes and sterols shows that while the animal and the lower plants (fungi, etc) contain only derivatives of lanosterol,[1a] the higher plants contain other triterpenes as well as lanos-

[1a] There is one exception to this statement, whale contains ambrein However, this triterpene is structurally distinct from all the others [L. Ruzicka and F. Lardon, *Helv Chim Acta* 29, 913 (1946)] and may be considered as formed by an unrelated enzyme

terol and its derivatives. It thus appears that the early life forms possess only the enzyme which cyclizes squalene to lanosterol and that later during the evolution of the plants, they acquired, for reasons unknown, an additional cyclization enzyme which folds the squalene chain to give a chair-form ring B during cyclization The various plant triterpenes would then be the result of subsequent minor changes in this new enzyme

C. Experimental Findings on the Cyclization of Squalene to Lanosterol

Two years after the original proposal by Woodward and Bloch, and by Dauben and Chaikoff and their co-workers that lanosterol is the product of squalene cyclization, Clayton and Bloch succeeded in demonstrating that lanosterol is a normal constituent of liver, that it is formed from acetate, and that it can be further converted into cholesterol (*41, 106*). Using labeled acetate it was further shown that *in vivo*, squalene and lanosterol, and some other compounds structurally related to lanosterol, were formed at a more rapid initial rate than cholesterol (*42*) If the animal were allowed to survive for an hour or longer after the injection of acetate, however, the radioactivity recovered in squalene or in lanosterol was greatly diminished The appearance and the disappearance of these compounds with time conform to a precursor-product relationship with cholesterol [11] Using this injection method, it was possible to prepare radioactive squalene and lanosterol for the study of their further transformations

It should be pointed out here that Schwenk *et al.* observed some years ago the formation from acetate of "high counting companions" and showed that these sterols are precursors of cholesterol (*43–46*) These compounds are undoubtedly the same as those obtained later by Bloch and his co-workers which were shown to contain lanosterol, agnosterol, and partially demethylated derivatives of lanosterol. We shall return to these compounds later on

Since radioactive squalene of fairly high specific activity can be prepared easily by the injection of labeled acetate to rats, there remained only one difficulty in studying the process of squalene cyclization, namely

[11] The precursor-product relationship between squalene, lanosterol, and cholesterol has been recently confirmed by a careful study of their rate of turnover [A V. Loud and N L R Bucher, *J Biol Chem* **233**, 37 (1958)] These results satisfactorily explained the fact that, from an animal given C^{14}-acetate, cholesterol may have a higher specific activity than squalene, *even though squalene is a precursor of cholesterol* These kinetic results, together with the now overwhelming mass of data supporting the direct conversion of squalene to lanosterol, and thence to cholesterol, establish beyond any doubt the role of squalene as an obligatory intermediate in cholesterol biogenesis *This point has been discussed fully by Popják (2).*

an *in vitro* system that would carry out only the conversion of squalene to lanosterol, but not the reaction before or after it. This difficulty was overcome when it was observed that rat-liver homogenate prepared under certain experimental conditions carried out the cyclization of squalene to lanosterol but converted neither acetate to squalene nor lanosterol to cholesterol (*107*). Using this preparation, and subsequently a hog-liver homogenate of the same properties, the cyclization of squalene was studied with the use of D and O^{18} as tracers. It was demonstrated that the over-all conversion occurs without any incorporation of proton or hydroxyl group from the water into lanosterol (*50*). The process has an absolute requirement for oxygen which becomes incorporated into lanosterol during the reaction. No intermediate between squalene and lanosterol could be demonstrated under aerobic or anaerobic conditions. These findings are in complete agreement with the scheme of Ruzicka and co-workers which requires (1) the nonexistence of any stable intermediate, (2) the electrophilic attack by some form of oxygen, and (3) the nonincorporation of proton from the medium into the product of cyclization. More recently, Cornforth and Popják and their co-workers (*52*) and Bloch *et al.* (*51*), using slightly different approaches, independently obtained experimental evidence that during the enzymic cyclization of squalene to lanosterol, there were two 1,2-methyl shifts, from C-14 to C-13 and from C-8 to C-14, again in agreement with the scheme of Eshenmoser *et al.* (*48*). These results thus furnished experimental evidence that the intermediary carbonium ion in the formation of lanosterol must have the ring B in the boat form as shown previously in Section IV,B.

An examination of the cofactors required for the cyclization of squalene showed that, besides oxygen, there is also an absolute requirement for TPNH, microsomes, and a soluble protein (or proteins?) (*107*). There are also indications of a requirement for metal ions. This reaction thus falls into a general group of reactions where the substrate is oxygenated by molecular oxygen in the presence of a reductant, usually TPNH or DPNH. These reactions may be represented by the following reaction



$$S + O_2 + RH_2 \rightarrow SO + R + H_2O$$

where S is the substrate, and RH_2, the reduced pyridine nucleotide or other reductants. Most, but not all of these reactions are multi-enzyme systems with complex cofactor requirements. The nature of the active oxygen is not known and the mechanism of these reactions is still totally obscure. We shall, therefore, not discuss further these very interesting reactions, but shall refer the reader to the extensive review by Mason on

these and other oxygenation reactions (*108*), and to the more recent papers by Kaufman (*109*) and by Tomkins *et al* (*110*) on the nature of some cofactors involved.

The liver enzyme system cyclizes squalene to lanosterol exclusively. As was pointed out earlier, there must be other squalene-cyclizing enzymes in plants For this reason, the liver enzyme system was designated squalene oxidocyclase I The absolute stereospecifity of this enzyme requires that the squalene molecules be held on the enzyme surface in a rigid and definite configuration. Since squalene is a hydrocarbon with no polar groups, the nature of squalene-enzyme interaction poses a particularly challenging problem Unfortunately, squalene oxidocyclase I is a particulate enzyme that has resisted all attempts at solubilization (*111*) Therefore it is not possible at present to study this problem.

V. Further Transformations of Lanosterol

A. Oxidative Demethylation of Lanosterol

The conversion of lanosterol to cholesterol requires the removal of the three angular methyl groups, the saturation of the double bonds in the side chain and between the C, D ring juncture, and the introduction of a double bond into the 5,6 position We shall discuss these in the order listed.

The first question that arises when one considers the removal of the angular methyl groups is the level of oxidation at which these groups are removed. In the family of biochemical reactions of one carbon transfer, one encounters the transfer of the one carbon unit at several levels of oxidation Thus, in the reactions involving methionine and choline, one has the transfer of a fully reduced carbon, namely, a CH_3 group. In the reactions involving the folic acids, one finds "active C_1" at the level of $-CH_2OH$ and $-CHO$. In the CO_2 fixation and decarboxylation reactions, including the reactions of carbamyl-phosphate and "active CO_2," the C_1 involved is in the fully oxidized state

Olson, Lindberg, and Bloch studied the conversion of lanosterol to cholesterol in rat-liver homogenate using lanosterol biosynthesized from methyl-labeled acetate (*53*). The lanosterol thus formed contains 18 atoms of C^{14} and, upon conversion to cholesterol, should lose three atoms of C^{14} and give 15 atoms of C^{14} in cholesterol. All the C^{14} eliminated in this transformation was recovered as CO_2. The radioactivity could not be trapped by the addition of formaldehyde or of an acceptor for a C_1 unit from the folic acid derivatives. Furthermore, for short periods of incubation, some acidic material was recovered, indicating the oxidation of the methyl groups to carboxyl groups. Although such intermediates were

not isolated and identified, there is no lack of biological precedence wherein angular methyl groups are oxidized to alcohol or aldehyde groups: such as in sojasapogenole (*112*) and aldosterone, etc It is thus entirely reasonable to conclude that these three methyl groups in lanosterol are removed by oxidation to carboxyl groups followed by decarboxylation. The cofactors required are again TPNH and O_2, thus indicating that the primary attack on the methyl groups is probably by direct oxygenation This is indeed the only way of oxidizing such angular methyl groups since, due to the absence of H on the adjacent carbon atom, it is impossible to carry out dehydrogenation

Of the three methyl groups that are eliminated, the one on C-14 of lanosterol appears to be the first to be eliminated Gautschi and Bloch studied a radioactive compound isolated from chromatography of the nonsaponifiable fraction of rat liver after injection of methyl-labeled acetate (*113*) From the derived ratio of radioactive CO_2 and cholesterol obtained when this compound is incubated with liver homogenate, it was established that this compound has only two methyl groups more than cholesterol, thus identifying it as a *nor*-lanosterol Chemical degradation revealed the presence of the side chain double bond between C-24 and C-25, the presence of the *gem*-dimethyl group on carbon 4 and the presence of another double bond between carbon atoms 8 and 9 This compound is thus undoubtedly 14-*nor*-lanosterol The position of the double bond between rings C and D is interesting From a biological reasoning that zymosterol is probably a normal precursor in the biogenesis of cholesterol (we shall come to this point in detail a little later), one would expect the *nor*-lanosterol to contain the double bond $\Delta^{8,9}$. Chemically, one would expect the presence of the double bond $\Delta^{8,9}$, being β to the carboxyl group at carbon 14, to facilitate the decarboxylation and to migrate position 8 to 14 as a result of the decarboxylation Thus, the chemical argument and the biological argument seem to be at variance with each other Experimentally, it was found that the double bond remained at its original position This decarboxylation is hence an exception to the general behavior of β-unsaturated acids which normally undergo double bond migration during decarboxylation. Thus we have in this reaction, a modification of the "normal" course of reaction by the enzyme This influence of the enzyme may be attributed either to the possible steric hindrance involved if the double bond were to migrate, or to the lack of a proton on the enzyme surface near the carbon 9 of the sterol so that a concerted β-protonation and decarboxylation cannot take place

The order in which the two methyl groups on C-4 are removed is not known Recently, the presence of 4-α-methyl-sterols have been reported

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Olson, Lindberg, and Bloch studied the conversion of lanosterol to cholesterol in rat-liver homogenate using lanosterol biosynthesized from methyl-labeled acetate (*53*) The lanosterol thus formed contains 18 atoms of C^{14} and, upon conversion to cholesterol, should lose three atoms of C^{14} and give 15 atoms of C^{14} in cholesterol. All the C^{14} eliminated in this transformation was recovered as CO_2 The radioactivity could not be trapped by the addition of formaldehyde or of an acceptor for a C_1 unit from the folic acid derivatives Furthermore, for short periods of incubation, some acidic material was recovered, indicating the oxidation of the methyl groups to carboxyl groups Although such intermediates were

not isolated and identified, there is no lack of biological precedence wherein angular methyl groups are oxidized to alcohol or aldehyde groups: such as in sojasapogenole (*112*) and aldosterone, etc It is thus entirely reasonable to conclude that these three methyl groups in lanosterol are removed by oxidation to carboxyl groups followed by decarboxylation The cofactors required are again TPNH and O_2, thus indicating that the primary attack on the methyl groups is probably by direct oxygenation This is indeed the only way of oxidizing such angular methyl groups since, due to the absence of H on the adjacent carbon atom, it is impossible to carry out dehydrogenation.

Of the three methyl groups that are eliminated, the one on C-14 of lanosterol appears to be the first to be eliminated Gautschi and Bloch studied a radioactive compound isolated from chromatography of the nonsaponifiable fraction of rat liver after injection of methyl-labeled acetate (*113*) From the derived ratio of radioactive CO_2 and cholesterol obtained when this compound is incubated with liver homogenate, it was established that this compound has only two methyl groups more than cholesterol, thus identifying it as a *nor*-lanosterol Chemical degradation revealed the presence of the side chain double bond between C-24 and C-25, the presence of the *gem*-dimethyl group on carbon 4 and the presence of another double bond between carbon atoms 8 and 9 This compound is thus undoubtedly 14-*nor*-lanosterol The position of the double bond between rings C and D is interesting From a biological reasoning that zymosterol is probably a normal precursor in the biogenesis of cholesterol (we shall come to this point in detail a little later), one would expect the *nor*-lanosterol to contain the double bond $\Delta^{8,9}$ Chemically, one would expect the presence of the double bond $\Delta^{8,9}$, being β to the carboxyl group at carbon 14, to facilitate the decarboxylation and to migrate position 8 to 14 as a result of the decarboxylation Thus, the chemical argument and the biological argument seem to be at variance with each other Experimentally, it was found that the double bond remained at its original position. This decarboxylation is hence an exception to the general behavior of β-unsaturated acids which normally undergo double bond migration during decarboxylation Thus we have in this reaction, a modification of the "normal" course of reaction by the enzyme This influence of the enzyme may be attributed either to the possible steric hindrance involved if the double bond were to migrate, or to the lack of a proton on the enzyme surface near the carbon 9 of the sterol so that a concerted β-protonation and decarboxylation cannot take place

The order in which the two methyl groups on C-4 are removed is not known Recently, the presence of 4-α-methyl-sterols have been reported

(*114–117*).[12] From this one might be tempted to argue that it is the 4-β-methyl group which is removed first. However, this argument is not necessarily correct because of the following observations: Using lanosterol, 14-nor-lanosterol, and zymosterol labeled in the 3 position with tritium, Bloch and co-workers demonstrated that the hydrogen on C-3 is lost in the conversion of lanosterol and 14-nor-lanosterol to cholesterol, but is fully retained when zymosterol is converted to cholesterol (*118*) This can be taken to indicate that in the transformation of the sterols containing 4-methyl groups, the 3-OH group must be oxidized at some stage to a ketone Chemically, the presence of a β-ketone group at C-3 would greatly facilitate the decarboxylation. In the case of the β-keto acid derived from 14-nor-lanosterol, the product of this decarboxylation would be the enolate ion of 4-methylcholesta-8,24-dien-3-one, which probably isomerizes to the ketone and is then reduced to the alcohol. Thus the stereochemistry of the methyl group in the sterol would be determined by the stereochemistry of the ketonization and not by the original stereochemistry of the methyl group

B CHANGES INVOLVING THE DOUBLE BONDS

Little is known about the transformation of zymosterol to cholesterol. The current knowledge of this phase of cholesterol biogenesis has been reviewed by Bloch recently (*1*) and little additional information has since been gained We shall therefore content ourselves with a hypothetical reaction sequence (Fig 14) and a brief account of the experimental facts from which the reaction sequence is deduced. To begin with, we may assume that zymosterol, but not dihydrozymosterol, is a normal intermediate. This is based upon the observation that liver homogenate prepared by the action of a Waring Blendor can convert only zymosterol, but not its dihydro derivative, to cholesterol (*119*). The isolation of desmosterol with many times the specific activity of the accompanying cholesterol from chick embryo after injection of C^{14}-acetate further suggests that the reduction of the side-chain double bond is the last step in cholesterol synthesis (*120*). On the other hand, T-labeled Δ^7-cholestenol (lathosterol) has been shown to be converted to cholesterol (*121*). Also, 7-dehydrocholesterol appears to be converted to cholesterol in the intestine (*122*). These results tend to indicate that the side-chain double

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the other 4-methylsterols in that it still retains both methyl groups at the C, *D* ring juncture Its presence in nature indicates that the 4-methyl group(s) can be removed prior to the removal of the methyl group on C-14.

bond is reduced before the changes in the sterol ring system are completed It is quite obvious that desmosterol, Δ7-cholestenol and 7-dehydrocholesterol cannot all be included in a single pathway from zymosterol to cholesterol Whether the two pathways illustrated in Fig. 14 are normally coexistent or whether they reflect a difference between the various tissues cannot be ascertained at the present time However, it may be pointed out here that all of these compounds have not been demonstrated

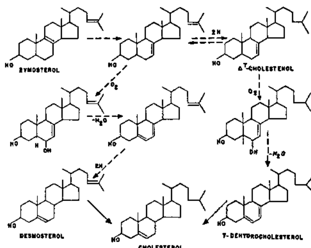


FIG 14 Hypothetical scheme of the transformation of zymosterol to cholesterol

in liver, quantitatively the most important organ in cholesterol biosynthesis Desmosterol was first isolated from chick embryo and more recently also from the skin The other two were both isolated from skin and Δ7-cholestenol appears to be a major constituent of skin sterol (*123–125*) As will be discussed later, there are some differences in the sterol metabolism in the skin and in the liver.

The dihydroxy-intermediates in the scheme are postulated to explain the requirement of oxygen in the conversion of zymosterol to cholesterol (*119*) Since a direct oxygenation reaction has been shown to be involved in many oxidation reactions of the sterols (cyclization of squalene, oxidation of lanosterol, and the biogenesis of the bile acids and the steroid hormones), it is postulated here that oxygen may be used directly to oxidize the Δ7-sterols to a dihydroxy compound which then dehydrates to give a $\Delta^{5,7}$-diene system Or, alternatively, these two steps may be achieved in one step by a dehydrogenation reaction In either case, the

oxidative reaction would explain the need for oxygen. This oxygen requirement, together with the ready conversion of 7-dehydrocholesterol to cholesterol, supports the hypothesis that, between zymosterol and cholesterol, there may be intermediates with the $\Delta^{6,7}$-diene system

C. Addition of the Carbon Atom(s) to the Side Chain by Plants

There is present in the plant kingdom, as well as in many of the lower forms of animals, a variety of derivatives of lanosterol or cholesterol with one or two additional carbon atoms on the C-24 of the side chain Among these compounds, two have been studied experimentally· eburicoic acid and ergosterol. Both of these contain one additional carbon on the side chain The source of this carbon atom has been identified as the general one-carbon pool of the cell It could arise from formate or methionine but not from acetate (*55–62*). Alexander and Schwenk (*60*) reported that with methionine labeled in the methyl group with both C^{14} and T, the ratio of C^{14}/T in the ergosterol isolated was the same as in the methionine used. Furthermore, the incorporation of the methyl group from methionine is not inhibited by the addition of aminopterin, a known inhibitor of the enzymes involved in the one-carbon metabolism involving folic acid derivatives The incorporation of formate into ergosterol, on the contrary, is strongly inhibited by this compound. It is thus established that the transfer of the one-carbon unit to the sterol does not involve any folic acid derivative and that the methyl group of methionine is transferred to the sterol without oxidation More recently, Parks reported that *S*-adenosylmethionine (*62*), commonly referred to as active methionine and which has been demonstrated to be the methyl donor in many biological reactions, is the actual methyl donor in the formation of ergosterol

The acceptor of the methyl group is still unknown When labeled methionine was used, the hydrocarbon fraction (squalene) recovered was not labeled, indicating that the methyl group is not introduced before the formation of the steroid nucleus (*59*). Using labeled squalene and sterols, it was shown that squalene and lanosterol, but not zymosterol, can be converted to ergosterol suggesting that the methyl acceptor is a sterol between lanosterol and zymosterol In the case of the C_{31} and C_{32} sterols and terpenes, the acceptor of the additional carbon atom(s) must of course be a C_{30} sterol or terpene. For a list of these naturally occurring derivatives of lanosterol and euphol, the reader is referred to the review by Jones and Halsall (*126*)

The use of the methyl group of methionine for the methylation of the side chain of a sterol furnishes a new type of reaction for active methio-

nine. It has been previously established that methionine can function as the source of the methyl groups attached to nitrogen (choline, creatine, etc.) or to oxygen (various sugar polymers), but this is the first demonstration of a transfer of the methyl group of methionine to another carbon atom.

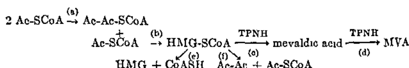
The methyl transfer from *S*-adenosylmethionine to form ergosterol makes it more interesting to consider the source of the C-24-ethyl (or ethylene) group in many sterols and terpenes. On paper, these compounds may be formed either by a C_2 transfer from ethionine (or "active ethionine") or by mechanisms entirely different from the addition of a C_1-unit to form ergosterol. If the latter is true, then there is at the present no basis for speculation on the mechanism. The other alternative faces the difficulty that ethionine has not been found in nature. However, in the many instances where the amino acid composition has been carefully determined (mammals, yeast, bacteria), no sterol or terpene with a two carbon substituent on the C-24 is synthesized. It would thus not be surprising that ethionine is not encountered in these organisms even if it is present in other organisms. In the author's opinion, the natural occurrence of ethionine has not been definitely ruled out and it is a distinct possibility that it may serve as the source of the C_2-unit in many sterols and terpenes.

VI. Control of Cholesterol Synthesis

In normal human, as well as in other species, the cholesterol level in blood is maintained at approximately 180 mg%, although this can be raised by the addition of large amounts of cholesterol in the diet. This relative constancy appears to be maintained by the feedback control of the rate of an early step (or some early steps) in the biogenesis of cholesterol in liver. The early experiments on the effect of various additions to the diet on the conversion of acetate to cholesterol have revealed that when cholesterol, squalene, or other sterols which can be converted to cholesterol is added to the diet, the rate of synthesis of cholesterol from acetate decreases.[11] Conversely, if the bile or the lymph is removed by the insertion of a fistula, thus causing a drainage of sterols, the rate of cholesterol synthesis from acetate is greatly stimulated (*127, 128*). Since the total incorporation of acetate into nonsaponifiable material is low-

[11] The literature on this effect has been reviewed recently by Gould and Cook (*6*), and so is not quoted here. It might be mentioned here that this feed-back inhibition functions primarily in the liver which is quantitatively the most important organ for cholesterol synthesis. In the other tissues, cholesterol synthesis is not, or is little, affected by cholesterol feeding.

ered after feeding of cholesterol, the site of this inhibition of the biogenesis of cholesterol from acetate must be prior to the formation of squalene. More recent experiments using labeled mevalonic acid have confirmed and extended this conclusion The feeding of cholesterol has little or no effect on the conversion of mevalonic acid to nonsaponifiable material (*129*). The site of this homeostatic control mechanism must, therefore, be located prior to the formation of mevalonic acid Since the activation of acetate to form acetyl-CoA is not inhibited by cholesterol feeding, it may also be concluded that the site of inhibition is after the formation of acetyl-CoA. Thus, the homeostatic control of cholesterol must be associated with one of the following steps



Several possibilities suggest themselves: (1) inhibition of reactions (a), (b), (c), or (d); (2) reduced supply of TPNH, and (3) increased activity of reactions (e) and (f).

Recently, Lynen and Bucher and associates (*130, 131*) have shown that in rats with modified rate of cholesterol synthesis from acetate (increased by injection of Triton and decreased by starvation), the rate of formation of acetoacetate and HMG-SCoA is not altered. The rate of reduction of HMG-SCoA to MVA, on the contrary, changes drastically in these animals This was not caused by the level of TPNH available, but was due to changes in the activity of HMG-SCoA reductase which parallels and is approximately equivalent to the *in vivo* rate of cholesterol synthesis. It thus appears that the rate limiting step in cholesterol biogenesis is the reduction of HMG-SCoA to MVA.[14]

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have postulated that the estrogens exert their effect via their functioning as co...
for a TPN-DPN transhydrogenase (*132*)

In the serum of patients suffering from congenital hypercholesterolemia, the level of cholesterol may rise to two or three times the normal value.[15] The cause of this is not known Indeed, it is not even certain whether this high cholesterol level is the direct effect of a genetic lesion or whether it is a mechanism to offset the effect of an unknown genetic lesion In these patients, the blood cholesterol level remains high even when the patients are kept on a cholesterol-free, low-fat diet It is thus clear that the genetic control is not simply on the rate of synthesis of cholesterol, but rather on the homeostatic cholesterol concentration in blood, or in other words, the gene probably governs the level of cholesterol in blood at which feedback inhibition is triggered

VII. Comparative Aspects of Cholesterol Synthesis

A Synthesis in Various Tissues

The above discussion on cholesterol biogenesis referred primarily to the synthesis of cholesterol in the liver Although liver is undoubtedly the major site of cholesterol synthesis, there are other tissues which also carry out the synthesis of cholesterol from acetate Thus the intestine has been shown to be extremely active in this respect The other tissues that have been examined for cholesterol synthesis are the adrenals, skin, nervous tissue, aorta, and the reproductive organs The synthesis of cholesterol from acetate in the adrenals and in the sex organs will be discussed in another chapter and will not be treated in detail here Suffice it to say that there are probably both a small, rapidly turning over pool and a large reserve pool of cholesterol in the adrenals The rate of conversion of acetate into cholesterol, which is rapidly converted to, and which can be measured in the form of the steroid hormones, is much less affected by ACTH than is the conversion of cholesterol from the reserve pool to the hormones (see Chapter 11 for references)

The intestine is very active in cholesterol synthesis (*133, 134*) Feeding of cholesterol has little or no effect on this activity. The pattern of sterol found in the intestine is similar to that observed in the liver with the exception that more Δ^7-sterols are present in the former (*135*)

The skin exhibits some interesting differences from the liver As was mentioned earlier, there is very little squalene (or other sterol precursors of cholesterol) in the liver In the skin, on the other hand, there is a much higher ratio of squalene to cholesterol In wool fat, there is a considerable

[15] The extensive literature on this subject has been reviewed recently by D Adlersberg and H Sobotka, in "Cholesterol" (R P Cook, ed), pp 375–425 Academic Press, New York, 1958

amount of lanosterol, dihydrolanosterol, agnosterol, and dihydroagnosterol (*136*); the latter two are probably artifacts resulting from the air oxidation of lanosterol and dihydrolanosterol (*137*) Δ^7-Cholesterol (*138*) and an as yet unidentified sterol (*139*) have also been reported to be present in considerable quantity in the skin of the rat. There is thus good evidence that in skin, unlike liver, considerable amounts of the intermediates of the synthesis of cholesterol are accumulated

Nervous tissue, the richest source of cholesterol, contains cholesterol that is metabolically inert Although growing nervous tissue (young animal or regenerating nerves) have been shown to synthesize cholesterol, normal nervous tissue carries out neither the synthesis nor the degradation of cholesterol (*133*, *140*). It is generally accepted that the cholesterol in the nervous system serves as a structural element and does not play any active part in the metabolism of these tissues (*141*). The synthesis of cholesterol in these tissues is governed only by the demand on structural units required for growth

B. Role of Squalene in the Liver of Sharks

We have stated previously that liver contains little squalene An exception to this general rule is found in the shark and related species of fish (*142*). Shark liver contains huge amounts of squalene in the autumn By contrast, the liver in early spring is smaller, less fatty, and contains only a small fraction of the squalene found in the fall. It appears that in these animals, squalene is stored in the liver as food reserve for the winter. Nothing is known about the pathway by which this reserve is utilized as an energy source

The [illegible] metabolism in the animal kingdom is [illegible] further [illegible] and all the steroid hormones In these transformations, the only reactions that are likely to yield energy efficiently are the oxidation of the side-chain carbons Considering [illegible] total oxidation of squalene can [illegible] of squalene via chole... [illegible] seems more likely that this large reserve of squalene in the livers of shark is degraded during the winter season by reactions not involving the formation of the cyclic sterols. At the present time, however, nothing is known about the manner in which it is utilized.

C. Sterol Synthesis in Different Species

Sterols are either synthesized in or required for the growth of all life forms with the exception of bacteria and viruses (if the latter are classified

as "living") (*143*) [16] In the plant kingdom, as we have mentioned earlier, a large number of triterpenes are also formed. Besides the presence of these terpenes, there is another difference between the plant and the animal kingdoms In the former, C-24-substituted sterols are encountered in all species examined, whereas in the animal kingdom, the 24-substituted sterols occur much more rarely. Existing information indicates that the vertebrates cannot synthesize these sterols In the invertebrates, 24-substituted sterols have been found, but the possibility has not been excluded that they may originate from the food (including plants) ingested by these organisms In the plant kingdom, on the contrary, the main sterols encountered are substituted on C-24 Indeed, except in the red algae (*144*), cholesterol itself has not been demonstrated in any quantity in plants, although derivatives of cholesterol are widespread in the higher plants (sapogenins and alkaloids)

This subject has been recently reviewed by Bergmann (*8*) to which the reader is referred

VIII. Excretion of Sterols in the Mammal

Sterols are excreted in the urine, feces, and the skin secretion Urine contains no C_{27} sterols but only the derivatives of the steroid hormones, and there in very small quantities The skin secretion contains considerable amounts of squalene as well as cholesterol (*145*), although less than that in the feces In the latter, a large number of sterols, steroid ketones, and bile acids have been demonstrated [see reference (*6*)], but one sterol, coprostanol, constitutes much more than half of the total. There is good evidence that this sterol, which is not absorbed by the intestines, is formed by the action of the intestinal flora on cholesterol (*146*, *147*). The mechanism of this transformation is still not clear. Gallagher and coworkers studied the conversion of cholesterol-3,D-4,C^{14} to coprostanol and found that not only is the deuterium on C-3 retained, but additional deuterium is found on C-6 (and possibly C-5) of coprostanol (*147*, *148*) These results rule out the old theory that cholestenone and coprostanone may be intermediates, and suggest a direct reduction of cholesterol to coprostanol

IX. The Bile Salts

A Introduction

The most common and best known bile acids are the hydroxylated derivatives of cholanic acid (C_{24}) excreted in the bile as the conjugate

[16] Using C^{14}-labeled acetate, the synthesis of sterols in the blue-green algae was demonstrated, thus showing that even in the lowest form of plants, sterols are present (*143*)

of either glycine or taurine However, in the bile of fishes, reptiles, and amphibians, one also finds taurine conjugates of hydroxylated coprostanic acids (C_{27}) and the sulfate esters of steroid alcohols (C_{27}) [see references (*149*, *150*)] Although these compounds differ greatly in chemical formula, construction of models of these compounds reveals one common structural feature the presence of several hydrophilic groups on the one side of the molecule and a large hydrophobic surface on the back. It is undoubtedly this common structural characteristic that enables these compounds to act as detergents and aid the emulsification and the absorption of lipid material from the intestine (*149*) The presence of these different bile acids in different species constitutes a very interesting aspect of comparative biochemistry which has been the subject of an excellent review by Haslewood (*149*)

In mammals, only bile acids with the cholanic acid skeleton are encountered The main bile acids vary from species to species Earlier work showed that these compounds are hydroxylated at carbons 3, 7, and/or 12 Recently, however, a large number of bile acids with a 6-OH group have been reported (*150–155*) The interpretation of the importance and the significance of these minor bile acids is complicated by the action of the intestinal flora on these compounds The origin of the bile acids was shown by Bloch, Berg, and Rittenberg in 1943 to be cholesterol (*156*) We shall attempt, in the following sections, to discuss briefly the chemical changes involved in the transformation of cholesterol to the bile salts and the secondary bacterial action on the latter in the intestine A glance at the structures of the bile salts and of cholesterol reveals that the following changes must take place (1) introduction of the OH groups on certain carbon atoms of the sterol nucleus, (2) inversion at C-3 to give the 3α-OH structure, (3) saturation of the double bond in cholesterol to give a *cis*-A,B ring juncture, (4) oxidation of the side chain, and (5) conjugation of the bile acids with taurine or glycine to give the bile salts These changes are listed here in the probable order in which they take place *in vivo*, although, as we shall see in the following sections, the exact sequence is still not yet established

B Introduction of Hydroxyl Groups

Bergstrom and his co-workers have shown that in the formation of cholic acid, the changes on the sterol nucleus are completed before the oxidation of the side chain [see references (*18*, *157*)] On the other hand, in certain bile acids, introduction of the hydroxyl groups on carbons 6 and 7 can be achieved after the degradation of the side chain Most of these results were obtained from *in vivo* experiments, except for the enzymic introduction of a 7-OH group into deoxycholic acid (*157–162*)

This reaction requires TPNH and oxygen and constitutes another example of biological oxygenation reactions which have been previously discussed in Section IV,C. The introduction of the hydroxyl group on C-12 is probably achieved in the same manner although experimental evidence is so far still lacking. Little is known about the origin of the 6-OH groups. Most of the bile acid with 6-OH groups also contain a hydroxyl group on C-7. Whether the presence of the latter is an essential structural feature for the introduction of the 6-OH group is not clear. The presence of the various stereoisomers (6α, 7α, 6α, 7β, 6β, 7α, and 6β, 7β) (*150–155*) indicates either a remarkable lack of specificity of the enzymes involved or the presence of a multitude of enzymes. The picture is further complicated by the possible bacterial action on these acids. In the case of deoxycholic acid, experiments with germ-free animals have shown conclusively that it arose entirely through the action of the intestinal bacteria on cholic acid (*163*). The origin of the various minor bile acids have not been examined as thoroughly and it is difficult to conclude at present which of these are formed by the secondary action of the bacteria.

C. Inversion at C-3 and Reduction of the Double Bond

These two processes are included in the same section, not because they are necessarily related, but because we know little about either of them. In all the known bile acids, these two processes have already been completed. The natural occurrence of lithocholic acid suggests that these reactions can take place prior to the introduction of the OH groups on carbons 7, 12, and 6. On the other hand, the major bile acids found in most mammals, are cholic, deoxycholic, and chenodeoxycholic acid, two of which (cholic and deoxycholic) cannot be formed from lithocholic acid [see reference (*157*)]. It has also been shown that 3α,7α-dihydroxycoprostane and 7α-cholesterol can be converted to cholic acid, whereas 3α-hydroxycoprostane cannot (*157*). These results indicate that in the major pathway of bile acid biogenesis, the inversion at C-3 and the reduction of the double bond probably occur after the introduction of the 7-OH group (Fig. 15).

D. Oxidation of the Side Chain

That the three terminal carbon atoms of cholesterol can be oxidized to CO_2 *in vitro* has been known for a long time [see reference (*6*)]. Indeed, the use of 4-C^{14} and 25-C^{14}-labeled cholesterol has shown that the oxidation of the side chain to form the bile acids is quantitatively the major catabolic pathway of cholesterol. The exact enzymic steps involved are, however, not clearly established.

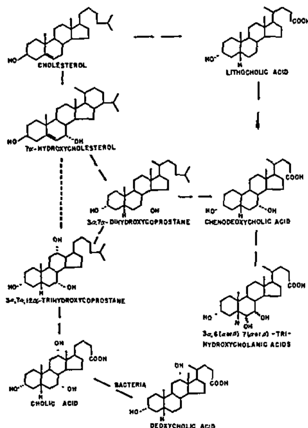


FIG 15 Formation of the bile acids from cholesterol

The first demonstration of the oxidation of cholesterol side chain by a cell-free system was reported in 1953 by Anfinson and Horning (*164*). Using rat liver homogenate, these workers showed that the C-26 of cholesterol is oxidized to CO_2. Later, the formation of 25-dehydro- (*165*), 25-hydroxy- and 26-hydroxycholesterol (*166*) was also demonstrated in rat and mice liver homogenates. Administration of these compounds to rats gave rise to bile acids (*166*) probably identical to those minor bile acids recently identified by Doisy and co-workers (*152–155*). The fact that no cholic acid was formed agrees with Bergstrom's theory that C-12-oxygenation cannot take place after the side chain has been oxidized (*157*) Based on this theory, the Swedish workers tested various compounds and found that $3\alpha,7\alpha$-dihydroxycoprostane, $3\alpha,7\alpha,12\alpha$-trihydroxycoprostane, and $3\alpha,7\alpha,12\alpha$-trihydroxycoprostanic acid are readily converted to cholic acid (*157, 167*) By combining these results an

incomplete scheme may be obtained for the degradation of the side chain during the formation of the 24 carbon bile acids (Fig 16).

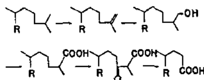
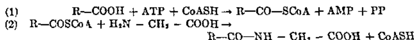


FIG 16 Oxidation of the cholesterol side chain in bile acid biogenesis

E. CONJUGATION OF THE BILE ACIDS WITH GLYCINE AND TAURINE

In the mammal, the bile acids are excreted entirely in the form of their glycine or taurine conjugate (*149*) The presence of the free bile acids in the feces and the intestine have been shown to be due to the intestinal flora (see next section). The amino acid in the conjugates is species specific Thus, the bile acids are excreted as the taurine conjugate in the chick, as the glycine conjugate in rabbit, and as a mixture of both in the rat

Experiments *in vitro* have shown that the formation of these conjugates of the bile acids involves the following steps (*168–172*):



(1) $R{-}COOH + ATP + CoASH \rightarrow R{-}CO{-}SCoA + AMP + PP$

(2) $R{-}COSCoA + H_2N - CH_2 - COOH \rightarrow R{-}CO{-}NH - CH_2 - COOH + CoASH$

An analogous reaction, substituting glycine for taurine, would lead to the formation of the taurine conjugates These reactions are carried out by the liver microsomes which have the same specificity for the amino acid as revealed by *in vivo* experiments (*172*)

F BACTERIAL ACTION ON THE BILE SALTS AND THE ENTEROHEPATIC CIRCULATION

We have already mentioned on several occasions that the intestinal flora are responsible for the occurrence of some bile acids in the feces So far, two transformations of the bile acids have been definitely shown to be due to these bacteria (1) the hydrolysis of the amide bond between the bile acids and the amino acids, and (2) the reduction of cholic acid to deoxycholic acid These have been demonstrated by the use of bile-fistula animals, germ-free animals or antibiotic-treated animals [see reference (*163*)] In all cases where the bile does not come into contact with the bacteria normally present in the intestines, unconjugated bile acids are absent In the germ-free or antibiotic-treated animals, deoxycholic acid is also not detected The hydrolysis of the conjugated bile

acids has also been demonstrated outside the animal body with pure strains of bacteria isolated from the feces (*173, 174*).

The bacterial reduction of cholic acid to deoxycholic acid has not been demonstrated *in vitro* although it occurs readily in the intestines of ordinary animals. *In vivo*, most of the deoxycholic acid formed is reabsorbed by the intestines. It is thus found in the bile of ordinary animals but not in the bile of germ-free animals. The absorption of the bile acids by the intestine is very efficient [see reference (*6*)], and only a small percentage escapes and is excreted in the feces. The absorbed bile acids are transported *via* the portal vein to the liver and then conjugated and reexcreted. The total time for such a cycle in rats, which do not have a gall bladder, is probably around two hours. This cycle has been named the enterohepatic circulation.

X. Concluding Remarks

The past few years have witnessed great progress in our understanding of the process of cholesterol biogenesis. Much confusion and ignorance, however, still exist in regard to several other aspects of cholesterol metabolism. The relationship between cholesterol metabolism and atherosclerosis, the role of unsaturated fatty acids and their cholesterol esters, the role of sterols in insects, protozoa, and plants. Much work has been done on the first two topics, but the picture is still very confused and full of controversies. About the role of sterols in insects, plants, and protozoa, little or nothing is known. For these reasons, these subjects have not been covered in this chapter. However, their omission should not be taken as equivalent to lack of importance. Indeed, the importance of understanding atherosclerosis is universally recognized, and knowledge concerning the function of sterols in the relatively simple unicellular organism may provide the answer to many basic questions in biochemistry.

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Metabolism of Steroid Hormones

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I. Introduction

This chapter deals with the means by which that group of compounds known as the "steroid hormones" are formed, distributed, and disposed of in the mammalian organism While steroid hormones occur in all vertebrates, detailed knowledge of their metabolism in other classes is too sparse to justify inclusion at this time The field of enzymic changes in steroid structure produced by adaptation of molds or bacteria to these substrates has become so large that recent reviews of this subject should be consulted (*1*, *2*).

Steroid hormones are formed in the mature cells of the gonads and in the adrenal cortex One group, the estrogens, is formed in the ovary during the development of mature follicles, and is responsible for the changes in the female secondary sex organs associated with estrus in lower mammals or the preovulatory phase of the menstrual cycle in primates Another group, the progestins or gestagens, is produced in addition to the estrogens by the corpus luteum during the period of diestrus in lower mammals or the secretory phase of the menstrual cycle in primates These affect the metabolism of the estrogen-stimulated uterus in such a way that a fertilized ovum will implant. The placenta which then forms also becomes an endocrine organ producing both

estrogens and progestins in some species, and thus assists in maintaining the proper uterine environment until parturition The interstitial cells of the testis form androgens which cause the growth and secretion of the male secondary sex organs and the development of male secondary sex characters

While the changes produced by these groups of compounds are particularly marked in the reproductive system, they have general effects, such as the influence on fat deposition in the female or the general increase in muscle protein in the male. Moreover, specific biochemical sequences such as fructose formation in the male secondary sex glands are dependent on their presence Their catalytic action, therefore, must either be exerted directly on a number of reactions by each type of molecule, or the effect must be on some factor which enters into a large number of reactions

The lipid-soluble active components of the adrenal cortex influence such widely different phenomena as the distribution and excretion of sodium and potassium ions, gluconeogenesis from protein, and the complex changes in damaged tissue called inflammation A number of chemically related compounds are produced by the cells of the adrenal cortex, each of which has differing proportionate influences on these processes Because of these differences one can assume that either each of the three major types of effect depends on a different type of reaction directly catalyzed by the steroid, or the difference in steroid structure determines the particular surface where a steroid may attach to catalyze a reaction common to these processes.

The steroid hormones are compounds closely related to cholesterol in ring structure, but have no more than two carbons in the side chain and possess more oxygen-containing groups. The conventions used in representation of structure and in numbering of carbons are those, therefore, which have already been explained in the chapter on sterols These will not be discussed further, except to call attention to the use of the dotted line to represent the steric position where the substituent is α or *trans* to the angular methyl groups at C-10 and C-13, and of a solid line to indicate the β or *cis* position

The parent saturated hydrocarbons are represented in Fig. 1. While a standard of nomenclature has been adopted by an international committee (*3*) certain terms are so fixed in the steroid literature that the attempt to substitute new trivial names has not been generally successful Usually the established common names have been used in this text.

The two 21-carbon compounds are 5α-pregnane (allopregnane) (I) and 5β-pregnane(pregnane) (II), the 19-carbon compounds are 5α-androstane (androstane) (III) and 5β-androstane (etiocholane) (IV),

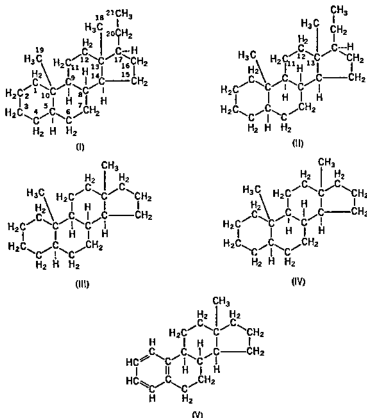
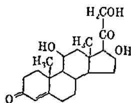


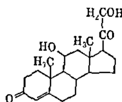
FIG 1 Parent hydrocarbons of the steroid hormones and metabolites (I), 5α-Pregnane(Allopregnane), (II), 5β-Pregnane(Pregnane), (III), 5α-Androstane(Androstane), (IV), 5β-Androstane(Etiocholane), (V), Estra-1,3,5(10)-triene The numbering of the carbon atoms is indicated in the formula for 5α-pregnane

while the only 18-carbon hydrocarbon involved is estratriene (V) Where a double bond exists between consecutively numbered carbons the lower number alone will be given, introduced between the first consonant and the vowel of the last syllable of the nuclear designation, thus pregn-4-en-20α-ol-3-one

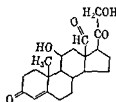
Before the biosynthesis and metabolism of the steroid hormones can be discussed we must determine what compounds we are considering A hormone may be defined as a compound produced in one cell and circulating in body fluids to other cells where it affects certain reactions catalytically. Until recently the evidence that a compound met these



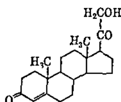
(VI)



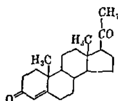
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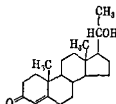
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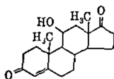
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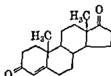
(X)



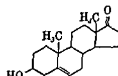
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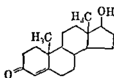
(XII)



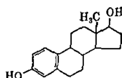
(XIII)



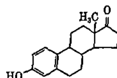
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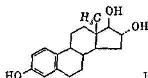
(XV)



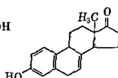
(XVI)



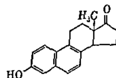
(XVII)



(XVIII)



(XIX)



(XX)

FIG 2 Steroid hormones secreted by the endocrine glands (VI), Cortisol(Pregn-4-ene-11β,17α,21-triol-3,20-dione), (VII), Corticosterone(Pregn-4-ene-11β,21-diol-3,20-

(IX), 11-
Pregn-4-ene-
rostenedione

criteria was indirect Substances could be isolated from specific tissues which, when injected, would relieve a biological deficiency caused by the removal of the tissue, but in most instances it was difficult to prove that the particular compound was actually secreted by the tissue, i e , that it was present in higher concentration in body fluids leaving the tissue than in those entering it Today micromethods make possible the measurement of hormones in fluids like blood, and many such compounds have been shown to appear in higher concentration in the blood leaving an endocrine tissue than in that entering it The compounds meeting all the criteria of steroid hormones are shown in Fig 2

The steroid compounds which have been shown to be in higher concentration in adrenal venous blood than in the arterial or peripheral venous circulation are listed in Table I,a Cortisol (VI), also called hydrocortisone and Compound F of Kendall, and corticosterone (Compound B of Kendall) (VII) appear to be the most important compounds which reverse the alterations in protein and carbohydrate metabolism produced by removal of the adrenal glands. Reich *et al* (*4*) first demonstrated that these compounds were present in the adrenal venous blood in concentrations greater than in the peripheral circulation. The ratio of the two differs among various species (*5*) and may change with age and variations in the degree and duration of stimulus by adrenocorticotropic hormone (ACTH) (*6*) No other compound having this type of physiological activity has been shown to be secreted in comparable amounts. Cortisone (XXI) is present in the peripheral blood and is not increased during flow through the adrenals more than during the transit through other tissues (*5*, *7*)

While the so-called "metabolic" hormones of the adrenal cortex have some influence on electrolyte balance in the vertebrates, and deoxycorticosterone (IX) is secreted in small amounts by the gland, the major influence on sodium and water balance is exerted by aldosterone (VIII), first isolated by Simpson *et al* (*8*) in 1953 Increased concentrations in adrenal venous blood of rats (*9*), dogs, monkeys (*10*), and humans (*11*) establishes its secretory nature

Progesterone (X), ordinarily thought of as an ovarian hormone, is also secreted by the adrenals of many, if not all, mammalian species, and probably plays a significant hormonal role in some It has been iso-

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(Androst-4-en-11β-ol-3,17-dione), (XIII), Androstenedione(Androst-4-ene-3,17-dione), (XIV), Dehydroepiandrosterone(Androst-5-en-3β-ol-17-one), (XV), Testos-

one), (XX), Equilenin(Estra-1,3,5(10),6,8-penten-3-ol-17-one).

lated from adrenal extracts (*12*), and is present in the adrenal venous blood of several species (*13*), including the nonpregnant human (*14*), in concentrations greater than that of arterial blood taken at the same time There can be little doubt, therefore, that the adrenal gland secretes this compound The functional significance in the different species and

TABLE I
COMPOUNDS DEMONSTRATED TO BE SECRETED BY THE GLANDS PRODUCING STEROID HORMONES

Compound	Structure No	Reference
a Compounds higher in adrenal venous blood		
Cortisol	VI	*4, 5, 7*
Corticosterone	VII	*4, 5, 7*
Aldosterone	VIII	*9–11*
11-Deoxycorticosterone	XIX	*11*
Progesterone	X	*13, 14*
11β-Hydroxyandrostene-3,17-dione	XII	*7, 16*
Androstene-3,17-dione	XIII	*7, 16*
Dehydroepiandrosterone	XIV	*7, 16*
Estrone	XVII	*17*
Pregn-4-en-20α-ol-3-one	XI	*15*
17α-Hydroxyprogesterone	XXII	*16*
11-Deoxycortisol	XXIII	*16, 97*
17α-Hydroxypregnenolone	XXIV	*131*
b Compounds higher in spermatic venous blood		
Androstene-3,17-dione	XIII	*18, 19*
Testosterone	XV	*18, 19*
c Compounds higher in ovarian vein blood		
Progesterone	X	*20, 21*
d Compounds higher in umbilical venous blood		
Progesterone	X	*23*
Pregn-4-en-20α-ol-3-one	XI	*23*
Pregn-4-en-20β-ol-3-one		*68*
Estriol	XVIII	*22*
Estrone (?)	XVII	*22*
17β-Estradiol (?)	XVI	*22*

the factors affecting output are not yet clear; but in the sheep, at least, where the secretion by the adrenals is high, ovariectomy seems to increase the output in nonpregnant females.

Pregn-4-en-20α-ol-3-one (XI), which is also formed by the ovaries and is progestational, appears to be abundant in the adrenal venous effluent of young calves but falls to low levels in that of the adult bovine (*15*). It may be associated with the changes going on in the adrenal during the early period of life

In addition, 11β-hydroxyandrostene-3,17-dione (XII), androstene-3,17-dione (XIII), and dehydroepiandrosterone (XIV), all androgenic compounds, have been found in the normal adrenal venous blood of certain species (*7*, *16*) There is evidence that increased amounts of estrone (XVII) are also present in the adrenal venous effluent (*17*)

Besides these hormonally active compounds a number of steroids, for which no role has thus far been definitely established, are frequently

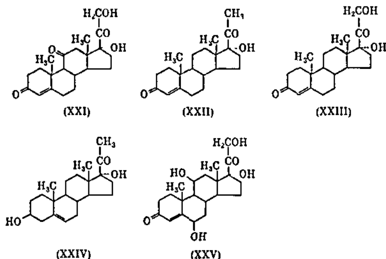


Fig 3 Steroids associated with adrenal function (XXI), Cortisone(Pregn-4-ene-

found in higher concentration in the blood of the adrenal vein than in that entering the gland Some of these, such as 17α-hydroxyprogesterone (XXII), 11-deoxycortisol (XXIII), and 17α-hydroxypregn-5-en-3β-ol-20-one (XXIV) (Fig 3), may simply represent the diffusibility of intermediates in the biosynthetic sequence, others, however, such as 6β-hydroxycortisol (XXV), would appear to require additional biosynthetic steps

We must realize the limitations in our recognition of hormonal substances· unless they produce marked biological alterations and are produced in quantity in only one tissue they would not thus far have been recognized as hormones It may be that certain more subtle biochemical

reactions are affected by these compounds which apparently have no hormonal function, and the gross disturbances produced by the absence of the other steroids have concealed them We cannot, then, unequivocally limit the steroid hormones to those which we have recognized in the past

The known secretory products of the testis are given in Table I,b The important androgenic hormones of the testis appear to be androst-4-ene-3,17-dione (XIII) (androstenedione) and testosterone (XV) While the relative concentrations of the two in the spermatic venous blood vary among species, both have been found to be higher in this fluid than in the general circulation, arterial and venous (*18, 19*)

The steroids shown to be present in ovarian vein blood in increased concentration are given in Table I,c Progesterone (X) can be definitely classified as an ovarian hormone, since it has been shown to be present in ovarian vein blood in greater concentration than in peripheral blood, moreover, it varies with the estrous cycle (*20, 21*) 17β-Estradiol (XVI) and estrone (XVII) probably fall into this classification although chemical estimation in ovarian vein blood has not been reported, estriol (XVIII), on the other hand, does not seem to be a normal secretory product of mammalian ovaries (*14, 22*).

The other tissue which has been amply demonstrated by indirect methods to be a producer of steroid hormones is the fetal placenta. Rigorous proof of secretion by this tissue would require that the levels in the umbilical vein be demonstrated to be higher than in either the umbilical artery or the maternal blood Such studies are rare, however, and most of the data on placental function have been obtained by comparing cord (umbilical vein) blood with the maternal blood only. When compounds are present in higher concentration in such blood than in the maternal circulation, the fetal organs as well as the placenta may be the sources of the steroid Under such circumstances the best circumstantial proof is the association of high concentrations of the steroid in the placenta with mechanisms for its synthesis in that tissue Progesterone and pregn-4-en-20α-ol-3-one (XI) meet these criteria in the human being [(*23, 24*) Table I,d] That estrogens are produced is also evident, but the secretory products are difficult to determine Free 17β-estradiol (XVI), estrone (XVII), and estriol (XVIII) have been isolated from the placenta (*25*) and the ability of placental tissue to synthesize these compounds has been demonstrated (*26–29*) In human umbilical cord blood, however, the concentrations of estrone and of 17β-estradiol are less than in the maternal blood while that of estriol is much higher (*22*) Most of the latter is conjugated as the glucuronide, and it is thought that this pattern may be the result of rapid conversion of the other two estrogens into

estriol by the fetal liver. The final answer can only be obtained by careful comparative analysis of blood from the umbilical vein and umbilical artery. During the latter half of pregnancy the placentas of the *Equus*, as distinguished from all other genera, appear to secrete equilin (XIX) and equilenin (XX) (*30*) since they are synthesized from acetate during that period

Dehydroepiandrosterone (XIV) in conjugated form is also present in higher concentration in cord blood than in maternal blood (*31*) but this probably represents a product of the fetal adrenal since this compound has not been found in significant amounts in the placenta

In summary, the hormones secreted by the various organs include those compounds which had previously been assumed to be important on the basis of isolation from the tissues Others, however, such as androstenedione, were not found originally in the glands which secrete them as major products In addition, other steroids are secreted for which we know no hormonal function as yet Whether these are incidental or actually play some undiscovered role remains for the experiments of the future to decide

These studies of secretory products have also demonstrated both quantitative and qualitative differences between species, and changes in proportion of different components, as well as in absolute rate, with age and internal environment. This is also true of the metabolic reactions which will be discussed Often observations have been made in only one species, frequently the human, and the possibility of species variation should always be kept in mind These differences, however, are quantitative rather than qualitative as far as the primary patterns are concerned

II. Biosynthesis of the Steroid Hormones

A. The Primary Pattern of Biosynthesis

The fundamentally conservative and consecutive nature of the evolutionary process in living organisms is well illustrated in the biosynthetic sequences by which the steroid hormones arise in the various tissues No matter what the source, or whether the ultimate product controls electrolyte balance, metabolic processes, or specific functions in the reproductive system, one basic biosynthetic pattern seems to underlie the formation of all In fact, the series of reactions by which these important regulators arise in the organism is simply an extension of the sequence involved in the formation of sterols by many cells of the body

The basic unit from which the steroid hormones, like the sterols, are formed is the acetyl group of acetyl CoA. When acetate labeled with C^{14} is incubated with slices or homogenates of any of the steroid-forming

tissues radioactive steroid hormones are obtained (*32–36*). Apparentl the synthesis is closely related to, if not identical with, that of cholestero The phosphate ester of mevalonic acid, (3,5-dihydroxy-3-methylvaleri acid) was reported to give rise to cholesterol (XXVIII) as well as estradio when incubated with homogenates of human testis (*37*) Moreover, th folding of the linked 2-carbon units must be similar to that of squalen to form cholesterol since within an error of not more than 8% all of C-2 of cortisol originates from C-2 of acetate while C-20 comes from C-1 of th acid radical (*38, 39*). Also, 10 carbons of androstenedione and 9 carbons o estrone biosynthesized from acetate come from the methyl group a required by the folding of squalene Thus either cholesterol or a compound in which squalene is folded in a similar fashion appears to be an obligatory intermediate in the synthesis of the steroid hormones from acetate

Whether cholesterol itself is an obligatory intermediate in the biosynthesis of the steroid hormones is still subject to debate Certainly cholesterol has been shown to serve as a precursor of any of the steroid hormones (*33, 40–42*) Enzyme systems which will split isocaproic acid from cholesterol leaving the C-21 steroid pregn-5-en-3β-ol-20-one (XXIX) have been demonstrated in the major steroid hormone-forming tissues (*43, 44*) Pregn-5-en-3β-ol-20-one (commonly simply called pregnenolone), as will be discussed further, can give rise to all of the steroid hormones Thus a clear course of synthetic reactions from cholesterol can be traced. The question is whether this is the only sequence of reactions or whether there may be some other compound formed from squalene which may also be converted to the steroid hormones without involving cholesterol

The evidence against cholesterol as an obligatory precursor consists of experiments where the relative labeling of cholesterol fractions is compared with that of the hormones when acetate-C^{14} has been used as substrate. Brady (*32*), in the first experiments demonstrating incorporation of acetate into testosterone, incubated testis slices of several species with acetate-1-C^{14} and found radioactive carbon in both cholesterol and testosterone; but while the amount of radioactivity in testosterone was increased approximately ten-fold when human chorionic gonadotropin was introduced into the incubation medium, no significant increase in radioactivity of the cholesterol could be found Hechter *et al.* (*45*) perfused bovine adrenal glands with blood containing either acetate-1-C^{14} or cholesterol-4-C^{14} and isolated cortisol from the perfusate. When acetate was the labeled precursor the cortisol had a much higher specific activity than either the plasma or tissue cholesterol. When labeled cholesterol was perfused, however, the specific activity of cortisol was lower than that of any of the cholesterol fractions. Bligh *et al.* (*46*) incubated hog adrenal homogenate with acetate-C^{14} and found incorporation of the

radioactive carbon into the adrenal steroids under circumstances where they were unable to identify any labeling of the tissue cholesterol Assuming that a considerable proportion of the cholesterol measured was in the same metabolic pool as the steroid hormones, these experiments would argue for alternative pathways of steroid hormone biosynthesis

It is highly probable, however, that the cholesterol of any one cell, let alone the cholesterol in a complex tissue like the testis, is not all in the same metabolic pool That pool size can be quite different from the total amount of a compound of this type in an organ, even when that compound is present in very small amounts, is illustrated by the experiments of Loud and Bucher (*47*), which show that only a small portion of the limited amount of total squalene in the liver cells is in equilibrium with the lanosterol formed therefrom Attempts have, therefore, been made to separate subcellular fractions in which the labeling of cholesterol from acetate-C^{14} might approach that of the steroid hormones formed, and show variations in the same direction when rate of incorporation into the hormones was changed Mason and Samuels (*48*) perfused dog testes with acetate-1-C^{14} at a constant rate before and after introduction of human chorionic gonadotropin (HCG) One testis was removed before HCG injection and the other after The testes were then homogenized in 0 25 *M* sucrose and fractionated centrifugally according to the classic methods of Schneider and Hogeboom (*49*) The digitonin precipitable material from every tissue fraction had much lower specific activity than that of the testosterone extracted from the blood, and did not show the marked increase in specific activity after HCG which was seen in the testosterone Even here, however, the amount of hormone formed was small in proportion to the cholesterol isolated and a small pool, rapidly turning over, might easily have been missed in one of the heterogeneous fractions which are separated by the centrifugal procedures Thus, while there is good reason to question cholesterol as an obligatory intermediate in the synthesis of the steroid hormones the definitive experiment has not yet been done

Cholesterol is a precursor, whether obligatory or not, and as has been pointed out, the adrenals and the reproductive tissues have enzymic systems which will split off the side chain as isocaproic acid, yielding pregnenolone (Fig 4) This type of reaction is hardly a one-step affair and, as in most cases in biological systems, intermediate stages of oxidation are apparently involved When cholesterol-4-C^{14} was incubated with adrenal homogenates and various possible intermediates were added as trapping agents the only one in which radioactivity was present after incubation was 20β-hydroxycholesterol (XXX); no C^{14} was present in the 22α-hydroxy-, 22β-hydroxy-, 24β-hydroxy-, 22-keto-, or 24-keto-

cholesterols (*50*). If a doubly oxygenated compound involving both the 20- and 22-positions is a *momentary* intermediate, it seems that 20β-hydroxylation must always precede the 22-oxidation.

Pregnenolone seems to play a central role in the biosynthesis of the steroid hormones. All tissues which form steroid hormones contain an enzyme system which will convert pregnenolone to progesterone (*51*). Progesterone so formed is readily converted into androstenedione and

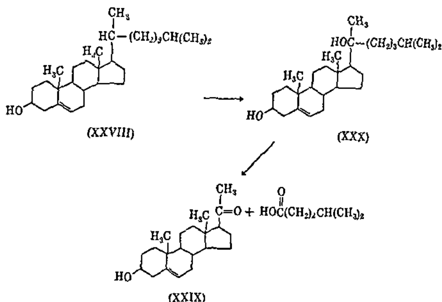


FIG 4 Conversion of cholesterol to pregnenolone Cholesterol (XXVIII) is probably first hydroxylated to 20β-hydroxycholesterol (XXX), which then yields isocaproic acid and pregnenolone (XXIX)

testosterone by testis tissue (*52*), into cortisol and corticosterone by adrenal perfusion (*53*), and to estrogens *in vivo* (*54*). Testosterone will be converted to estradiol, and androstenedione to estrone, by placental, ovarian, and testicular tissue (*27*, *55*, *56*). These experiments indicate that pregnenolone and progesterone, like cholesterol, can give rise to every steroid hormone thus far identified.

Some workers have argued that there may be alternative pathways from cholesterol to the 19-carbon steroids However, when a mixture of cholesterol-C^{14} and pregnenolone labeled with H^3 was incubated with adrenal slices the ratio of C^{14} to H^3 in the 19-carbon steroids dehydroepiandrosterone and androstenedione was the same, within the error of method, as that in the 21-carbon compounds where the obligatory nature

of pregnenolone as an intermediate had already been demonstrated (*57*) Thus it appears that the major, if not the sole, pathway of dehydroepiandrosterone and androstenedione biosynthesis from cholesterol in the adrenal is via pregnenolone.

On the basis of perfusion experiments Hechter and Pincus postulated a definite sequence for the various oxidations and hydroxylations involved in the conversion of progesterone to the cortical steroids (*58*) (Fig 5). Progesterone could undergo either 17α-hydroxylation or 21-hydroxylation If the latter occurred first, the former was not possible thereafter, only 11β-hydroxylation could occur since deoxycorticosterone yielded only corticosterone (*59*) If 17α-hydroxylation occurred first, 21-hydroxylation could follow, giving rise to 11-deoxycortisol (XXIII) This compound could then be hydroxylated to cortisol by the 11β-hydroxylating system Corticosterone, however, was not converted to cortisol or vice versa, and 11β-hydroxyprogesterone was a poor precursor for the cortical steroids (*60*) They, therefore, postulated that a compound hydroxylated in either position 21 or 11 could not attach to the 17-hydroxylating enzyme Either the unsubstituted progesterone or the 17α-hydroxyprogesterone (XXII) could serve as substrate for the 21-hydroxylase, but if the compound had been hydroxylated at carbon 11, it would not serve as substrate for either the 17- or the 21-hydroxylases. While these distinctions are not so rigid as originally stated, this appears to be the preferred sequence, i e , the compounds postulated as substrates for each step are the best substrates, although not the exclusive ones As with other types of enzymes, there can be sufficient attractive forces between the active sites on the enzyme and groups in various steroids to permit some interaction, but the ones originally postulated fit best.

The introduction of the 17α-hydroxyl group into progesterone weakens the bond between the 2-carbon side chain and the nucleus Slaunwhite and Samuels (*52*) found that whenever they obtained radioactive androstenedione and testosterone after incubating progesterone-4-C^{14} with testis homogenates they also obtained 17α-hydroxyprogesterone-4-C^{14} as long as any of the original substrate remained When 17α-hydroxyprogesterone was the substrate the two C_{19} steroids were formed rapidly but no progesterone was found If either progesterone-21-C^{14} or 17α-hydroxyprogesterone-21-C^{14} were incubated with these homogenates, the androstenedione and testosterone spots were not radioactive but the radioactivity was found in a steam-volatile acid, later established as acetic acid (*61*) The splitting of the side chain requires oxygen as well as TPNH as cofactor and the first product is androstenedione The latter compound is then reduced to testosterone in the testes of most species. The latter reaction has been demonstrated to be reversible (*62*).

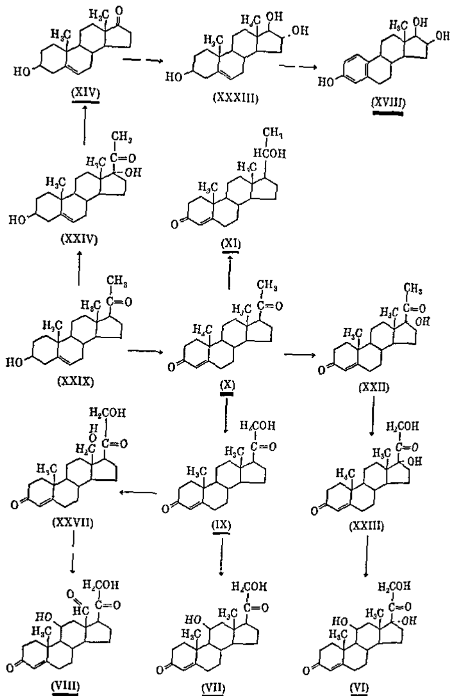
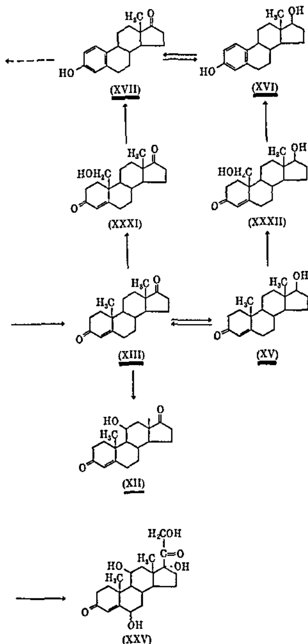


FIG 5 General pattern of steroid hormone biosynthesis from pregnenolone For compounds (VI) to (XXIX) refer to previous figures (XXVII), Pregn-4-ene-18,21-diol-3,20-dione, (XXXI), 19-Hydroxyandrostenedione(Androst-4-en-19-ol-3,20-dione), (XXXII), 19-Hydroxytestosterone(Androst-4-ene-17β,19-diol-3-one);



(XXXIII), 16α-Hydroxyandrostenediol (Androst-5-ene-3β,16α,17β-triol) ——→ indicates more than one step involved The formula numbers of important hormones are underlined

As mentioned earlier, testosterone can be converted to estradiol and androstenedione can be metabolized to estrone by any of the steroid hormone-forming tissues. Any double bond formation involving carbon 10 would require the displacement of the 19-methyl group, since carbon 10 is a tertiary carbon The most logical method of removal would be by oxidation This appears to be the mechanism. Adrenal and placental homogenates will convert androstenedione to 19-hydroxyandrostenedione (XXXI) (63, 64). Placenta and ovarian follicular tissue can convert 19-hydroxyandrostenedione or 19-norandrostenedione to estrone (26). Since, as already pointed out, the formation of estrone from androstenedione has been demonstrated for all the steroid-forming tissues, 19-hydroxylase and decarboxylating systems are probably present in all Thus a consecutive series of oxidations and hydroxylations has been traced which lead to such diverse molecules as cortisol and estrone.

Before turning to the individual enzymic reactions, a moment might be taken to consider the question of the similarities and differences between the various steroid hormone-forming cells Already attention has been called to the common course of the reactions by which acetate is used to synthesize cholesterol, and cholesterol is converted to pregnenolone and progesterone In addition, the adrenal has been shown to carry out any of the reactions described except, possibly, the conversion of androstenedione to testosterone Homogenates of bovine ovaries form 17α-hydroxyprogesterone and androstenedione as intermediates in the biosynthesis of estrone, as shown by trapping experiments (65) These are not artifacts of the incubation technique but actual intermediates formed in the ovaries, since Zander (66) was able to isolate both compounds from either follicles or corpora lutea of human beings. Homogenates of normal testes from several species will produce small amounts of deoxycorticosterone and 11-deoxycortisol from progesterone (67). Thus, except perhaps for the 11β-hydroxylase, which thus far has only been clearly demonstrated in the adrenal cortex and in interstitial cell tumors of the testis, the differences between the enzymic activities of the different tissues secreting steroid hormones are quantitative rather than qualitative. The primordia of all these organs arise in the region of the germinal ridge, and the similarity of pattern may be related to this fact

The quantitative differences, then, are in most cases the factors which give the individual steroid-forming organs their distinct hormonal character The adrenal cortex is distinguished by the high activity of 21-hydroxylase, a relatively low side-chain-splitting activity and little, or no, 17β-hydroxysteroid dehydrogenase It has a very active 11β-hydroxylase in the mitochondria, and thus far seems unique among normal tissues in this respect.

The testes contain relatively high side-chain-splitting and 17β-hydroxysteroid dehydrogenase activity, although the latter is much less in the rodents than in other mammals which have been studied While the systems required for aromatization are present, the activity of the 17α-hydroxylase and side-chain-splitting systems is relatively much greater than in ovarian tissues 21-Hydroxylase activity is very low but measurable Thus the major products are the C_{19} steroids

The ovarian follicle has relatively low 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase and side-chain-splitting activities in proportion to the 17β-hydroxysteroid dehydrogenase, 20-hydroxysteroid dehydrogenase, and aromatizing systems In the human and sheep the 20α-dehydrogenase appears to dominate while in the mare and cow the 20β-dehydrogenase seems the major one (*68, 69*) When the conversion to a corpus luteum occurs this seems to be associated with a marked increase in 3β-hydroxysteroid dehydrogenase activity with relatively little change in the other systems In the follicular phase, therefore, estrogens are the major products, while during the luteal phase progesterone, pregn-4-en-20-ol-3-ones, and estrogens are secreted

The placenta is unique among endocrine organs in that its existence is temporary and its function, the maintenance of a proper metabolic state in the uterine endometrium, is local The human placenta has high 3β-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase, aromatizing and 17β-estradiol dehydrogenase activities but little 17α-hydroxylase This leaves unsolved the question of the route by which C_{19} steroids might be formed for aromatization Since a major product of the human placenta, aside from progesterone, is estriol the observations of Ryan may be pertinent He found that while he obtained estrone and estradiol from incubation of androstenedione or testosterone with placental homogenates he found no estriol, but when he incubated androst-5-ene-3β,16α,17β-triol (XXXIII) he obtained estriol (*70*) It may be that there is a route thus far undiscovered via 16α-hydroxylation which is the major course of estrogen formation in the placenta.

B. The Individual Enzyme Systems

The biosynthetic sequence as outlined in Fig 5 covers the major products which have been identified, but this does not mean that each step is a simple one involving only one enzyme and no other intermediates The various enzyme systems known to be involved are listed in Table II, together with their intracellular localization and their cofactor require-

TABLE II
ENZYME SYSTEMS INVOLVED IN STEROID HORMONE BIOSYNTHESIS

Enzyme system	Cofactors	Reaction	Cell fraction
3β-Hydroxysteroid dehydrogenase	*DPN*[c] or TPN	Reversible	Microsomes
Δ^4-Δ^5 Steroid isomerase	None	Irreversible	Supernatant
17α-Hydroxylase[a]	TPNH + O_2	Irreversible	Microsomes
21-Hydroxylase[a]	TPNH + O_2	Irreversible	Microsomes
17α-Hydroxysteroid hydroxylase (side-chain-splitting)[a]	TPNH + O_2	Irreversible	Microsomes
11β-Hydroxylase[a]	TPNH + O_2	Irreversible	Mitochondria
18-Hydroxylase[a]	TPNH + O_2	Irreversible	—
17β-Hydroxysteroid dehydrogenase	DPN or *TPN*	Reversible	Microsomes
17β-Estradiol dehydrogenase	*DPN* or TPN	Reversible	Supernatant
20α-Hydroxysteroid dehydrogenase	DPN or *TPN*	Reversible	Supernatant
19-Hydroxylase[a]	TPNH + O_2	Irreversible	—
Aromatizing system[b]	TPNH + O_2	Irreversible	Microsomes

[a] These enzyme systems probably include three catalytic units an oxygen activator, a steroid activator, and an electron transfer enzyme

[b] This is probably a multiple system but no intermediates beyond 19-hydroxy-androst-4-ene-3,17-dione have been identified

[c] Italic letters indicate the preferred coenzyme

they have been measured, the Michaelis constants and maximum velocities of the steroid hydroxylases and dehydrogenases have been low, indicating a strong binding between the steroid and the enzyme with relatively slow breakdown of the enzyme-steroid complex.

1 *The Hydroxysteroid Dehydrogenases*

a 3β-HYDROXYSTEROID DEHYDROGENASE This enzyme system is present in all tissues synthesizing steroid hormones (*51*) It is contained in the microsomes, and all efforts to solubilize it have been associated with large losses of activity (*71*) DPN is the preferred hydrogen acceptor, and the transfer of hydrogen is direct The enzyme is strictly specific for the 3β-OH configuration, no 3α-OH steroids are affected Like the soluble β-dehydrogenase which Marcus and Talalay induced in bacteria (*72*), the microsomes will act on either C_{19} or C_{21} steroids providing an oxygen function is located on C-17 or C-20 However, elimination of ring *D*, even though a ketonic group is on C-13, will prevent any dehydrogenation of a 3β-hydroxy group The presence of a double bond in ring A is not essential (*73*)

b 17β-HYDROXYSTEROID DEHYDROGENASE. TPN is the preferred cofactor for this enzyme the most important function of which is to convert androstenedione to testosterone. This enzyme differs from the 17β-estradiol dehydrogenase in preferred substrate, preferred cofactor,

and localization in the cell Unlike Talalay's soluble β-hydroxysteroid dehydrogenase induced in *Pseudomonas testosteroni* (74) this enzyme does not seem to be the same as the 3β-dehydrogenase, either in preferred cofactor or distribution For instance, its activity in rat testes is low while 3β-hydroxysteroid dehydrogenase activity is high (*51*, *75*, *76*). It is a reversible system favoring the hydrogenated form at pH 7 4 and room temperature In view of this reversibility the relative concentrations of TPN and TPNH will affect the proportion of androstenedione and testosterone in the system

c. 17β-ESTRADIOL DEHYDROGENASE Whenever 17β-estradiol is found in the reproductive tissues estrone is also found because of the presence of a 17β-dehydrogenase which is specific for steroids having a benzene ring It differs from the previously described dehydrogenases in not being associated with a cellular particle as well as in its substrate specificity It resembles the 3β-dehydrogenase in utilizing DPN and DPNH better than TPN or TPNH Like the other dehydrogenases, however, the reaction will go on at a measurable rate with either nucleotide (77)

An exhaustive study of substrate specificity and kinetics by Langer *et al* (78) indicates that, since a benzene ring and not specifically a phenolic group is important, the attraction between that part of the substrate molecule and the enzyme is probably due to hydrophobic forces The steric and positional specificity indicate three-dimensional importance at the C-17 position, and the marked influence of distant groups in the steroid would lead to the view that the whole surface interacts with the enzyme During a fifty-fold purification of the enzyme the rate of oxidation of testosterone and 19-nortestosterone remained 5% of that of 17β-estradiol This would indicate that these compounds with a single double bond in ring A do interact sufficiently for an occasional molecule to undergo hydrogen transfer, and since the absence of the angular methyl group at C-10 did not affect the interaction, lack of the planarity characteristic of the benzene ring rather than steric interference of the methyl group was responsible for failure of the C_{19} 17β-hydroxysteroids to be good substrates

This enzyme, like the other dehydrogenases, appears to possess essential sulfhydryl groups, and is inactivated by Cu^{++}, Hg^{++}, and Fe^{+++} but activated by Zn^{++}

2 *Steroid Hydroxylases*

a. C-6, C-11, C-17, C-18, C-19, and C-21 HYDROXYLASES These hydroxylations resemble one another in that all require TPNH and atmospheric oxygen (*63*, *79–84*)

The only one of these which has been obtained in soluble form in any

concentration is the 11β-hydroxylase; as a consequence, most of the work on mechanism of action has been done with this enzyme In view of the similarities in TPNH and O_2 requirement, however, and the resemblance to nonsteroidal hydroxylases, the conclusions regarding the character of the 11β-hydroxylase probably apply to the whole group First, there is no exchange of oxygen (*83*, *84*) or hydrogen (*85*) with the ions of water; the exchanges must take place at the enzyme surface. Second, the reaction appears to be a direct replacement of a steric hydrogen with hydroxyl, since a sterically oriented hydrogen is replaced with a hydroxyl having the same steric position (*86*) Third, H_2O_2, if it is involved, must be quite localized and protected, since added H_2O_2 does not accelerate, and catalase does not inhibit, the reaction Azide does not inhibit, and cyanide does not inhibit except at high concentrations, but diethylthiocarbamate is a strong inhibitor (*84*) This would indicate that while a metal is probably involved, the metalloenzyme differs from cytochrome *c*, cytochrome oxidase and the copper enzyme of the phenolase complex.

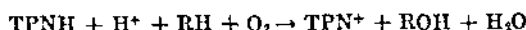
This is supported by the studies of Ryan and Engel (*87*) on the 21-hydroxylase of adrenal microsomes The resistance to azide and cyanide was similar to that of the 11β-hydroxylase In addition CO was found to produce a light-reversible inhibition and cytochrome *c* gave strong inhibition which could be reversed by cyanide Since cytochrome *m* of microsomes shows similar characteristics they suggest that it may be involved in the electron transfer

The steroid hydroxylating systems, like the phenol oxidases, are apparently composed of more than one enzyme Tomkins and co-workers (*88*) have been able to obtain active 11β-hydroxylation with partially purified soluble extracts of adrenal mitochondria, but three different heat-labile nondiffusible components and one diffusible heat-stable substance must be supplied in addition to TPNH and oxygen. Perhaps one enzyme forms the hydroxyl radical from atmospheric oxygen while a second activates the substrate and the third transfers the electrons from TPNH to form H_2O. The complex is probably associated in the mitochondrion and thus functions more efficiently in particulate form.

As previously mentioned, the various hydroxylases resemble each other wherever cofactor needs and inhibitors can be compared It seems probable, therefore, that they are all complexes made up of an iron-porphyrin enzyme of the cytochrome *m* type associated with an enzyme which activates a specific carbon in the steroid, and perhaps also a further component involved in the final electron transfers This may explain some of the difficulties in solubilizing what was assumed to be a single catalyst. In view of the occurrence of all these hydroxylases, with the exception of the 11β-hydroxylase, in the microsomes one wonders if the

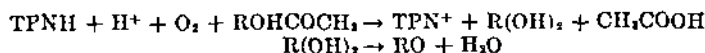
same radical-forming and electron-transferring components may not serve several steroid activating proteins

b SIDE-CHAIN SPLITTING SYSTEM This enzyme appears to be a hydroxylase, but it differs in certain ways from the other steroid hydroxylases Whereas in all the others there is a direct replacement of hydrogen by an activated OH, and the over-all reaction can be written:



$$TPNH + H^+ + RH + O_2 \rightarrow TPN^+ + ROH + H_2O$$

the ratio between moles of 17α-hydroxyprogesterone split and moles O_2 used is 1 1 (*61*), and the reaction can then be represented



$$TPNH + H^+ + O_2 + ROHCOCH_3 \rightarrow TPN^+ + R(OH)_2 + CH_3COOH$$
$$R(OH)_2 \rightarrow RO + H_2O$$

Like the other hydroxylases the rate of reaction is not increased by addition of low concentrations of H_2O_2 and it is not decreased by catalase It shows the same resistance to cyanide, but is not inhibited by carbon monoxide Ferrous ion in low concentrations is beneficial

3 Δ^5-*Hydroxysteroid Isomerase*

Since the conjugated double bond system of the Δ^4-3-keto structure is more stable than the Δ^5-3-keto arrangement, after dehydrogenation of the 3β-hydroxysteroids by the 3β-hydroxysteroid dehydrogenase migration of the double bond will occur at a slow rate nonenzymically in media which furnish the ionic environment and the cofactors necessary for the dehydrogenation The rate, however, is less than that which occurs in the presence of homogenates, and Talalay and Wang (*89*) showed that an isomerase is present in the cytoplasm which catalyzes the double bond transfer without requiring any known cofactor During this transfer, unlike that involving hydrogen or hydroxyl ions, there is no interchange of hydrogen with that of the water, so it must be a direct transfer of hydrogen on the surface of the enzyme (*90*)

4 *Aromatization of* C_{19} *Steroids*

The mechanisms of aromatization of the steroid nucleus in the mammalian organism have not been well worked out As noted earlier, the reproductive and adrenal tissues have been demonstrated to have a 19-hydroxylase, and 19-hydroxyandrostenedione will be converted to estrone (*26*, *63*, *64*) The whole system is present in placental microsomes (*91*) and TPNH and oxygen are the only cofactors necessary If the microsomes are well washed, only 17β-estradiol is obtained from testosterone, and estrone from androstenedione—evidence that the placental

microsomes are relatively free of the 17β-hydroxysteroid dehydrogenase just discussed. The intervening steps have not been worked out, but while both *Corynebacterium simplex* and *Pseudomonas testosteroni* convert 19-nortestosterone to estradiol and estrone (*92, 93*), the 19-nor compound was a poor substrate for aromatization by placental microsomes; it seems, therefore, that if it is an intermediate, it cannot readily exchange with the steroid in solution.

C. Abnormal Biosynthesis

Abnormal balances of the biosynthetic enzymes can occur either congenitally or because of malignant change. Malignancies of steroid-forming cells show many different patterns of steroid biosynthesis. Apparently during the malignant change the enzyme synthesizing systems may be disorganized in a chance fashion (*94*).

The most striking example of congenital biosynthetic enzyme deficiency is congenital adrenal hyperplasia with virilism. The disease usually results from a partial deficiency of the 21-hydroxylase; if the deficiency were complete the individual would die soon after birth because of complete lack of both cortisol and corticosterone (*95, 96*). Since the 3β-hydroxysteroid dehydrogenase and 17-hydroxylase systems are normal, 17α-hydroxyprogesterone and progesterone accumulate. Large amounts of 17α-hydroxyprogesterone escape into the blood, at the same time the side-chain-splitting system is kept saturated, and increased amounts of androstenedione and 11β-hydroxyandrostenedione are secreted. The secreted compounds undergo reduction and conjugation in the liver and increased amounts of the resulting 17-ketosteroids and pregnane-3α, 17α,20α-triol appear in the urine.

In another form of the disease in which hypertension accompanies virilization the deficiency appears to be in 11β-hydroxylation (*97*). Large amounts of the reduction product of 11-deoxycortisol (XXIII): pregnane-3α,17α,20α,21-tetrol, and that of deoxycorticosterone: pregnane-3α, 21-diol-20-one, are excreted while high levels of 11-deoxycortisol are found in the plasma. Here the enzyme deficiency has caused accumulation of three intermediates: 11-deoxycortisol, 17α-hydroxyprogesterone, and progesterone. In addition to increased side-chain splitting the higher concentrations of progesterone in the presence of the 21-hydroxylase lead to greater deoxycorticosterone synthesis.

Thus, in view of the common route of biosynthesis, it is not surprising that tumors or congenital abnormalities in one organ which secretes steroid hormones may lead to symptoms of excess production of the hormones characteristic of another steroid-forming tissue, even when the primary defect is an enzyme deficiency.

III. Transport and Distribution

The approximate normal concentrations of the various steroid hormones which have been measured in the human peripheral circulation are given in Table III They are expressed as μg per 100 ml plasma because

TABLE III
NORMAL CONCENTRATIONS OF STEROID HORMONES AND METABOLITES IN HUMAN PLASMA

Compound	State	μg per 100 ml plasma		
		8–9 A M [a]	5 P M [a]	Late pregnancy
17-Hydroxycorticosteroids[b]	Free	15 5(7–25)	7 3(4–12)	10–46
17-Hydroxycorticosteroids[c]	Conj	7–18	—	—
Cortisol	Free	12(7–25)	—	—
Corticosterone	Free	1 1(0 4–2 0)	—	—
Dehydroepiandrosterone[d]	Conj	48 6(32–60)	39 7(21–51)	—
Androsterone, males	Conj	18 0(11–25)	15 8(12–20)	—
Androsterone, females	Conj	13 3(8–15)	8 0(7–10)	—
Progesterone, females	Free	0 5	—	9–45
Estrone, females	Total	0 06–0 22	—	2 6–10 3
Estradiol, females	Total	0 07	—	1 4–3 0
Estriol, females	Total	0 15–0 30	—	4 3–17 5
Pregnanediol, females	Conj	10–20	—	30–75

[a] Where values are given for morning and afternoon the same subjects were used for both times Where values have been determined at night they continue to fall slowly till about 2 A M The group consisted of 6 men and 5 women where no distinction on the basis of sex is made there was no significant difference

[b] Free 17 hydroxycorticosteroids are equivalent to the sum of cortisol and cortisone

[c] *Conjugated 17-hydroxycorticosteroids include all metabolites having the 17α,21-diol-20-one structure in the side chain.*

[d] According to recent unpublished work, the values for dehydroepiandrosterone and androsterone may be too low because of incomplete extraction

at these concentrations the red cells contain very little hormone All those steroids which have been found to increase in concentration in the blood as they pass through the ovaries, adrenals, or testes are extractable with organic solvents and are not covalently linked with other groups. Yet their concentrations in extravascular body fluids and in urine in relation to plasma are less than would be expected for nonpolar freely diffusible molecules The interaction of steroids with plasma proteins seems to be an important factor in increasing the solubility of steroids in aqueous media and in determining this difference in distribution

At high concentrations of steroid the interaction with serum albumin accounts for the solubility effect within the limits of experimental error (*95–100*) Albumin has a high capacity for binding steroids 5% human

serum albumin is not saturated when testosterone is present at 1000 times the concentration in normal adult spermatic vein blood. Apparently the electrostatic component of the forces involved is so small that the binding with respect to solubility in water decreases with polarity *(101)* Schellman *et al.* *(102)* have studied the binding of testosterone with serum albumin and found that it depends on a large positive entropy change; they have, therefore, concluded that "configurational adaptability" as the result of local relaxation of tertiary folding of the protein must play a large part.

If this is true, it must be related to the influence of the phenolic groups of tyrosine When pH is increased from 2 to 11 there is a slow, steady increase in binding by serum albumin, but between pH 11 0 and 11 6 there is a marked decrease which is reversible *(103)* The data obtained would fit with the postulate that one molecule of testosterone is released when two tyrosine hydroxyls of the albumin are ionized Ketenization of the phenolic groups greatly reduced the binding, but iodination had no significant effect Thus the steroid molecules must be held between the tyrosine side chains of the serum albumin in such a way that the iodine atoms on the adjacent carbons do not cause steric interference *(104)*

As long as methods of estimation depended on colorimetric or spectrophotometric techniques only high concentrations relative to normal levels of plasma steroids could be studied With the synthesis of C^{14}-labeled steroids, however, much smaller amounts could be measured This also made possible the determination of distribution and turnover at the low concentrations normally present in blood When human volunteers were injected with amounts of cortisol sufficient to raise plasma levels to approximately ten times the normal 8 A.M values, extrapolation of the removal curves to zero time indicated that the hormone was distributed throughout approximately 80% of total body mass, assuming uniform concentration When tracer doses were given at this time, however, the distribution was equivalent to approximately ⅓ of the body mass At midnight endogenous levels are about ⅓ those of the early morning, and when tracer doses were given the apparent distribution volume was only about ¼ body mass *(105)* Thus diffusibility seemed to increase as levels were higher Daughaday and Kozak *(106)* offered the first proof that this was due to the presence of a small amount of a nondiffusible substance which bound cortisol much more strongly than albumin but was saturated at relatively low levels They carried out equilibrium dialysis experiments at very low concentrations of steroid. Below 0 3 μmol/liter 99% or more of the cortisol was bound at 4°, between this concentration and 0.6 μmol/liter the ratio of bound to diffusible steroid

changed rapidly and above this latter concentration approached th which would be expected from the known binding constant of seru albumin (Fig. 6) Electrophoresis studies indicated that the strong binding protein molecule when combined with cortisol moves as α_1-globulin but is different from the thyroxine-binding protein (10 Unlike albumin this fraction combines with cortisol more strongly th with corticosterone

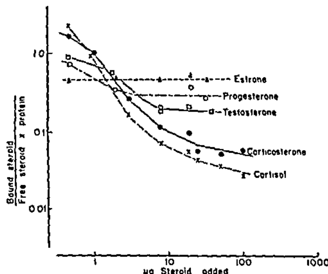


Fig 6 Changes in the binding affinity of normal human plasma for cortisol-4-C corticosterone-4-C[14], progesterone-4-C[14], testosterone-4-C[14], and estrone-16-C[14] the presence of increasing amounts of total steroid in a standard dialysis equilibri system containing 10 ml of plasma and 80 ml of buffer From W. H Daughad *J Clin Invest* 37, 511 (1958)

Slaunwhite and Sandberg (*108*) have carried out a more detai study of the binding protein which they have called "transcortin." dialyzing plasma against serum albumin at essentially the same conc tration as that in the plasma the effect of this protein was offset. Un such circumstances they were able to show that the ability of compone *of plasma other than albumin to bind steroids is particularly marked* cortisol, falls off considerably with corticosterone, is very small w testosterone and unmeasurable with estrone

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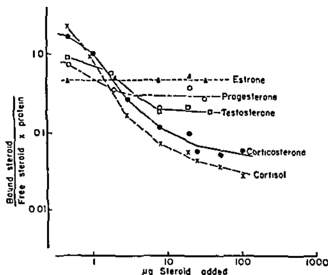


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compete and the effectiveness of the competition with cortisol is related to the degree to which they are bound when present as the sole steroid These results argue for a single specific protein or group of proteins being involved Until transcortin is isolated, however, a final answer cannot be obtained

This also raises the question of the identity of the proteins which bind thyroxine with those which bind steroids In both cases the amount of binding increases during pregnancy. As noted, Daughaday considered that the cortisol-binding protein moved somewhat differently in his electrophoretic system than would have been expected for the thyroxine-binding protein; this difference, however, might simply be due to the difference in influence of the bound groups on electrophoretic mobility. Until simultaneous binding and competition studies have been carried out there will be no clear-cut answer.

IV. Metabolism of the Steroid Hormones

Once they enter the blood, the average half-lives of the steroid hormones are relatively short Alterations in the steroid molecule itself, as well as covalent combination with other compounds, balance the flow

TABLE VI
HALF-LIVES OF VARIOUS STEROID HORMONES IN THE BLOOD OF THE ADULT HUMAN BEING

Steroid	Half-life Period B (minutes)[a]	Half-life Period C (minutes)	Reference
Cortisol	60-90	—	*113, 114*
Cortisol	84	—	*115*
Corticosterone	60	—	*115*
Aldosterone	25	—	*115*
Testosterone	11	100	*116*
Progesterone	20	90	*117*
Estrone	90	—	*118*

[a] Period A is the period of rapid equilibration and the slope is not given

of hormone from the secretory gland The major tissue involved is the liver, although reactions involving carbons 6, 17, or 20 can occur in a number of, perhaps in most, tissues In Table VI are given some average half-lives of steroid hormones as determined by the injection of "tracer" doses of radioactive hormone into relatively normal human beings Data obtained by the injection of large amounts of hormone have not been included because the studies with cortisol and progesterone indicate that as the level goes up the half-life increases (*105*) This is probably because

a greater proportion of steroid is freely diffusible when these abnormally high levels are achieved due to the saturation of the specific binding protein. In the table the data for cortisol are derived from the injection of doses which would not raise the total blood concentration more than 10%, in the case of the other hormones, however, where the endogenous levels in normal nonpregnant individuals are below the limits of accurate measurement, the so-called "tracer" doses may have significantly altered the concentrations in blood In any case, the average half-life would not be longer at normal endogenous levels

In the table the initial rapid fall in plasma concentration which each hormone shows is not included because this seems to represent primarily the period of distribution equilibrium Where two half-lives are given, as in the case of testosterone and progesterone, both appear to be related to the balance between diffusion and metabolic removal These are nonpolar steroids which seem to enter the fat depots rather readily Probably the first slope is that dependent primarily on the relation between diffusion in aqueous media and hepatic metabolism, the second probably represents the return of the hormone from the fat depots to the circulation and its consequent removal.

A Extra-hepatic Metabolism

While hepatic changes are quantitatively the most important, many tissues can bring about oxidative or reductive changes in the steroid hormones Eviscerated rats metabolize steroids, as shown by the conversion of progesterone to pregn-4-en-20α-ol-3-one or cortisol to cortisone (*119–121*) These reactions may be related to mechanisms of hormonal action, they may be due to extremely low concentrations of specific enzymes, and again they may simply represent the degree to which the steroids act as substrates in enzyme systems which are primarily carrying on oxidation or reduction of other substrates at much higher rates

Mammalian red cells, themselves, contain one or more enzymes which will oxidize a 17β-hydroxy group to a 17-ketone Estrone is formed from 17β-estradiol or androstenedione from testosterone In the case of the estrogens the reversibility of the system has been demonstrated (*122–124*). The rates of these reactions are so low in comparison with other changes in the steroid molecules, however, that they play a very minor role in the over-all metabolism of the steroids

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male sex organs than in other tissues (*140, 141*) The material isolated proved to be androstenedione rather than testosterone, although in the blood no trace of the diketone could be found Androstenedione is also formed by incubation of human prostatic tissue with testosterone and DPN (*142*), the system is reversible Thus these tissues, also, contain a 17β-hydroxysteroid dehydrogenase which may play a role in hormonal action

The presence of small amounts of hydroxysteroid dehydrogenase and 20-keto reductase activities in such tissues as muscle, heart, skin, and intrascapular brown fat (*135, 136, 143–146*) raises the question of whether these may simply be the result of the steroids acting as rather weak substrates for other alcohol dehydrogenases present in the cells The answer can be obtained only when pure enzymes are studied

B Hepatic Metabolism

The metabolic reactions which have thus far been discussed would not account for the rapid turnover of the steroid hormones indicated by their half-lives Moreover, in most animals the metabolic products of the steroid hormones appear in the urine or feces as conjugates of glucuronic or sulfuric acid No such conjugation occurs in hepatectomized or in eviscerated animals (*120, 121*) The liver is the major site of both the changes in the steroid molecules and their combination with acidic groups They leave the liver, therefore, in more water-soluble form and are more easily eliminated from the body.

The enzymes involved in hepatic metabolism of the steroids can be grouped into hydrogenases, dehydrogenases, and hydroxylases In this organ the major reactions are reductive, particularly in ring A Apparently a hydroxyl group in position 3 on the completely saturated ring is most easily acted upon by the enzymes which conjugate the steroids with glucuronic and sulfuric acid The benzene ring of the estrogens cannot be reduced by the hepatic enzymes, but in the case of estriol the 16α-hydroxy group readily conjugates with glucuronic acid The phenolic hydroxyl of the other estrogens readily conjugates with sulfuric acid The over-all changes which have been demonstrated to take place with each of the major groups of steroid hormones are given in Figs 8–13

1 Δ^4-*Hydrogenases*

As indicated in the figures, both the 5α and 5β isomers of the various steroids are formed on the reduction of the Δ^4 double bond This is not due, however, to lack of steric specificity in the enzymes involved Not only are there distinct Δ^4-5α hydrogenases and Δ^4-5β hydrogenases but the two types are differently distributed within the liver cell the Δ^4-5β

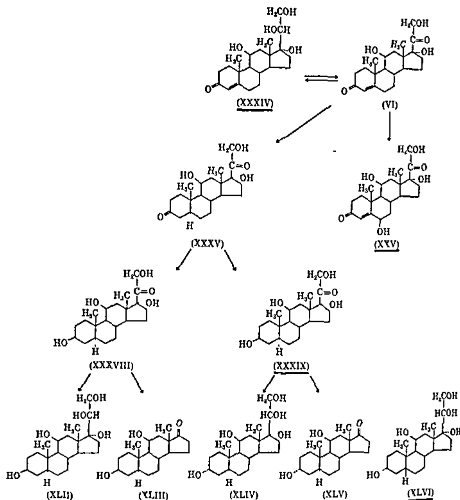
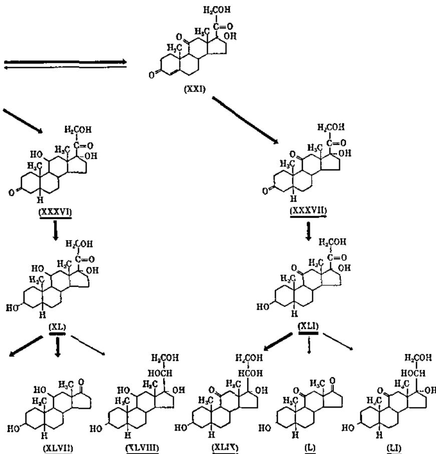


FIG 8 Metabolic pathways of cortisol (XXXIV), Pregn-4-ene-11β,17α,20β,21-tetrol-3-one, (XXXV), 5α-Pregnane-11β,17α,21-triol-3,20-dione, (XXXVI), 5β- [illegible]

17α,20β,21-pentol, (XLIII), 5α-Androstane-3β,11β-diol-17-one, (XLIV), 5α-Pregnane-3α,11β,17α,20α,21-pentol, (XLV), 5α-Androstane-3α,11β-diol-17-one(11β-Hydroxyandrosterone), (XLVI), 5β-Pregnane-3α,11β,17α,20α,21-pentol(Cortol);



(XLVII), 5β-Androstane-3α,11β-diol-17-one(11β-Hydroxyetiocholanolone), (XLVIII), 5β-Pregnane-3α,11β,17α,20β,21-pentol(β-Cortol), (XLIX), 5β-Pregnane-3α,17α,20α,21-tetrol-11-one(Cortolone), (L), 5β-Androstan-3α-ol-11,17-dione(11-Ketoetiocholanolone), (LI), 5β-Pregnane-3α,17α,20β-21-tetrol-11-one(β-Cortolone) Many of the reductions of ketone groups which are not represented by double arrows are reversible in isolated systems, but because the ring A-saturated-3-alcohols are readily conjugated with glucuronic and sulfuric acids the flow *in vivo* is in the direction indicated Heavy arrows indicate the major routes of metabolism in the human being, formula numbers which are underlined are those of compounds found in human urine

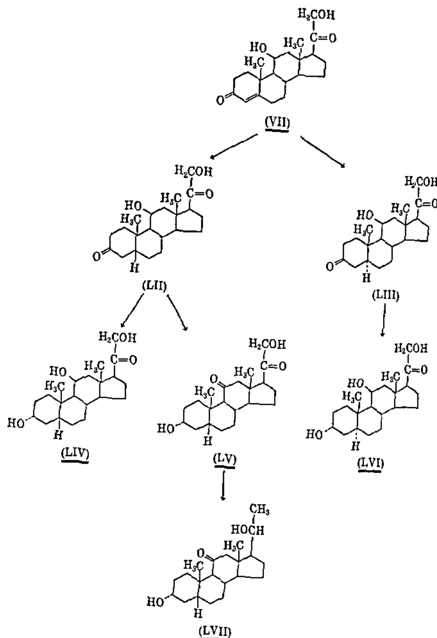


FIG 9 Metabolism of corticosterone (LII), 5β-Pregnane-11β,21-diol-3,20-dione, (LIII), 5α-Pregnane-11β,21-diol-3,20-dione, (LIV), 5β-Pregnane-3α,11β,21-triol-20-one(Tetrahydrocorticosterone), (LV), 5β-Pregnane-3α,21-diol-11,20-dione, (LVI), 5α-Pregnane-3α,11β,21-triol-20-one, (LVII), 5β-Pregnane-3α,20β-diol-11-one For explanation of symbols see legend for Fig 8

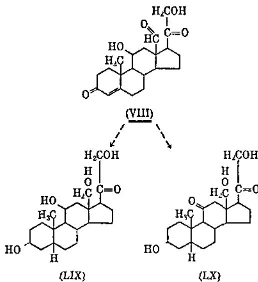


FIG 10 Metabolism of aldosterone (LIX), 5β-Pregnane-3α,11β,18,21-tetrol-20-one, (LX), 5β-Pregnane-3α,18,21-triol-11,20-dione Dashed arrows indicate that there must be multiple intermediate steps

hydrogenases are in the supernatant while the Δ^4-5α hydrogenases are in the microsomes (*146, 147*) Curiously enough, according to Forchielli *et al* (*148*) the female rat liver has only the microsomal Δ^4-5α hydrogenase while the male liver contains both

The supernatants obtained after high speed centrifugation of rat liver homogenates will reduce the Δ^4 double bond of a wide range of C_{19} and C_{21} steroids Tomkins found however that as he carried out successive steps of enzyme purification, using cortisone as substrate, the ability to reduce other steroids was lost. In his purest preparations even as closely related a steroid as cortisol would not be reduced (*147*) Fractions prepared by different procedures gave different ratios of activity with respect to various steroids Apparently there is a whole series of highly specific Δ^4-5β hydrogenases in the liver supernatant

The same seems to be true of the Δ^4-5α hydrogenases of the liver microsomes (*149*) While it was impossible to carry out purification steps in the same way as with the soluble enzymes, it was found that the ratios of the rates of reduction among various steroids varied from one enzyme preparation to another and in the same preparation with aging and other treatment This could only be true if different functional groups were involved. Such a high degree of specificity must require the inter-

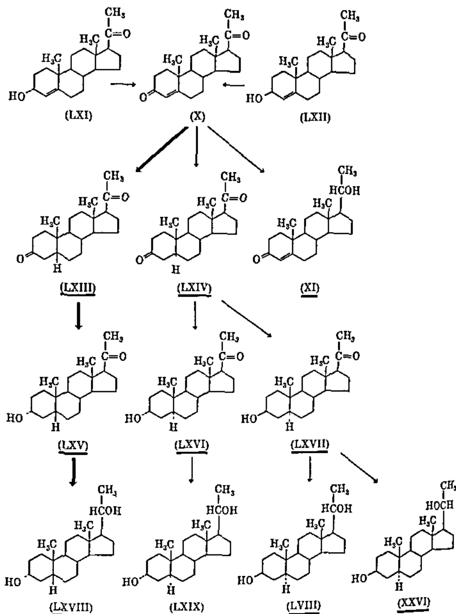


Fig 11 Metabolism of progesterone (LXI), Pregn-4-en-3α-ol-20-one, (LXII),

symbols as in legend for Fig 8

action of multiple parts of the steroid molecule with the enzyme molecules The hydrogenases require TPNH as cofactor and the transfer of hydrogen appears to be direct The systems appear irreversible

2 *Hydroxysteroid Dehydrogenases*

The major proportion of the secondary alcohols formed on the reduction of the 3-ketone group has the 3α configuration Both soluble and microsomal 3α-hydroxysteroid dehydrogenases have been described (*150, 151*) Unlike the Δ^4 hydrogenases, these enzymes appear to be relatively nonspecific as far as structure outside of ring A is concerned. Tomkins found that over a wide range of purification the relative activity toward such widely different substrates as 5β-pregnane-17α,21-diol-3,11,20-trione and 5β-androstane-3,17-dione remained constant These enzymes can utilize either DPN or TPN and the system is reversible Talalay has suggested, therefore, that they could function as nucleotide transhydrogenases It is unlikely, however that they perform such a function in the liver to any extent since, as previously pointed out, the alcohol group on the reduced ring is readily conjugated with glucuronic or sulfuric acids and the reaction, therefore, tends to run in only one direction

The small amount of 3β-hydroxysteroid dehydrogenase activity may be due to the relative nonspecificity of the 17β-hydroxysteroid dehydrogenase Talalay found that his bacterial preparations would reduce both 3- and 17-ketones Until the purified liver enzyme has been tested on more substrates a definite answer cannot be given

The 17β-hydroxysteroids are rapidly converted to 17-ketones by a soluble enzyme from the liver This 17β-hydroxysteroid dehydrogenase uses either DPN or TPN but DPN is the preferred cofactor Its K_m is $3\ 3 \times 10^{-5}$ moles per liter (*152*)

Another microsomal enzyme is an 11β-hydroxysteroid dehydrogenase which catalyzes the readily reversible reaction cortisol $\rightleftharpoons$ cortisone (*151*) Like the 3α-dehydrogenase this enzyme can utilize either DPN or TPN A 20β-hydroxysteroid dehydrogenase is also present in microsomes According to Recknagel, it utilizes TPNH preferentially as hydrogen donor but will reduce the 20-keto group with DPNH at $\frac{1}{6}$ the rate (*153*). 6β-Hydroxysteroids have been converted to 6-ketosteroids by mouse liver microsomes (*154*) While no fractionations were carried out, Breuer *et al* (*155, 156*) incubated 16-ketoestrone and 16-keto-17β-estradiol with liver slices and obtained both the 16α- and 16β-hydroxy derivatives This would indicate that there are also 16α- and 16β-dehydrogenases present

Thus dehydrogenases seem to be present for all those positions in the steroid hormone molecule where the ketone-secondary alcohol interconversion could occur The dehydrogenases, while stereospecific and

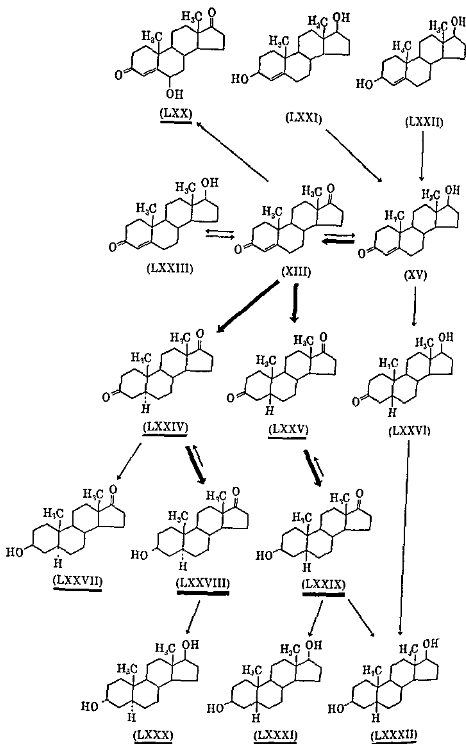


Fig 12 For descriptive legend see opposite page

although not with equal effectiveness Usually the systems are readily reversible, and the net direction of conversion will depend on the relative concentrations of both substrates and cofactors at any one time

3 *Hydroxylases in the Liver*

As a whole, the steroid hydroxylases of the liver do not seem to play so important a part in steroid metabolism as the dehydrogenases and hydrogenases 6β-Hydroxylases which will act on estrogens have been demonstrated in mouse liver microsomes (*154*) Rat liver homogenates will hydroxylate 11-deoxycortisol metabolites in this position, and 6β-hydroxy compounds were reported after perfusion of rat livers with deoxycorticosterone, testosterone, and androstenedione (*157*) 2β-Hydroxylation was also reported in perfusion experiments (*158*) The significance of these in hepatic metabolism is unknown at present. Side-chain-splitting has been demonstrated both with homogenates and by perfusion, using 11-deoxycortisol, cortisol, or cortisone (*159–161*) Since 11-oxysteroids of the C_{19} series are known to be metabolic products of cortisol (*162, 163*), this may explain their formation Where cofactor requirements have been studied, these hydroxylases, like those of the biosynthetic tissues, require TPNH and atmospheric oxygen, and are essentially irreversible

An important reaction in estrogen metabolism which occurs in the liver is the conversion of estrone and 17β-estradiol to estriol A 17β-estradiol dehydrogenase readily catalyzes the interconversion of estradiol and estrone, and the assumption was that estrone was converted to estriol by a hydration reaction In recent studies, however, it was found that injected 16α-hydroxyestrone, which normally occurs in small amounts in the urine, was largely excreted as estriol without any increase in estradiol or estrone (*164*) Since the formation of estriol in liver homogenates

Fig 12 Metabolism of androstenedione and testosterone (LXX), Androst-4-ene-6β-ol-3,20-dione(6β-Hydroxyandrostenedione), (LXXI), Androst-4-ene-3β,17β-diol, (LXXII), Androst-4-ene-3α,17β-diol, (LXXIII), Androst-4-en-17α-ol-3-one(Epites-

legend for Fig 11

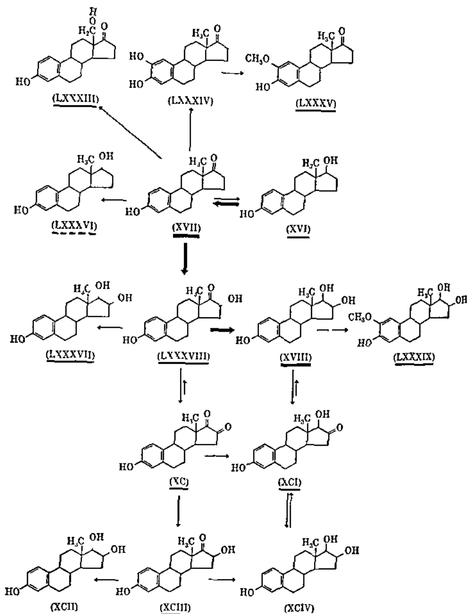


FIG 13 Metabolism of the estrogens (LXXXIII), 18-Hydroxyestrone, (LXXXIV), 2-Hydroxyestrone, (LXXXV), 2-Methoxyestrone, (LXXXVI), 17α-Estradiol, (LXXXVII), 16α,17α-Estriol, (LXXXVIII), 16α-Hydroxyestrone, (LXXXIX), 2-Methoxy-16α,17β-estriol, (XC), 16-Ketoestrone, (XCI), 16-Keto-17β-estradiol, (XCII), 16β,17α-Estriol, (XCIII), 16β-Hydroxyestrone; (XCIV), 16β,17β-Estriol Symbols as in legend for Fig 8

requires atmospheric oxygen, it is now postulated that t
the conversion of estrone to estriol is 16α-hydroxylatio
reduction of the 17-ketone group by the 17β-estradiol deh

4 *Conjugation Reactions*

While the various reactions already discussed lead as a polar steroids, the products are still not very hydrophilic a be readily displaced from the plasma proteins and filtere kidneys They are largely excreted, both in urine and in b ronisides or sulfates The conjugation is carried out in the liv tinal wall by mechanisms similar to those for other cyclic phenols Glucuronic acid is transferred from uridine diphosph acid to the steroids by specific enzymes with the formation of phosphate (*165*) The sulfate is transferred from adenosine-3'- 5'-phosphosulfate (*166*) by an enzyme present in the liver s (*167*) Most steroids are largely excreted as the glucuronisides, tion is dehydroepiandrosterone which is excreted largely as th The glucuronisides are readily filtered by the kidney without reab the clearance being similar to that of creatinine (*168*) The sulfa ever, are cleared much more slowly (*131*) As previously mentio may account for the high level of conjugated dehydroepiandr in the plasma in spite of low production

C Over-all Metabolism

1 *Evidence of Other Reactions*

The routes by which the major portion of the steroid metabolit excreted differ among mammalian species The carnivores, herbivore rodents excrete most of the steroid metabolites by way of the bile makes a study of the structure of the metabolites difficult, since microorganisms of the gut can cause serious alterations. Where st have been made on the products collected in the bile of animals with fistulas the compounds found after injection of radioactive steroid mones have usually been the same as those in human urine The prima the horse, and the guinea pig excrete a major proportion in the urine, in distinctly different patterns

Most of the information on the metabolic products which are excret has been obtained from the human being While small amounts metabolites are excreted in the feces, the urine represents the maj route of elimination A minor portion in the urine is unconjugated, largel the original hormones filtered out from the freely diffusible portion i the plasma The greater part, however, occurs as the glucuronisides and

sulfates In the figures illustrating over-all metabolism the compounds which have been isolated from the urine have been underlined. Most of the urinary products can be accounted for by the hepatic enzymes which have been described. There are a few, however, which are still unexplained by the known enzymes.

Among the most important of these are the 16-hydroxylated steroids As pointed out earlier, the formation of estriol in the liver seems from indirect evidence to involve 16-hydroxylation of estrone followed by reduction of the 17-ketone, yet no liver fraction has been clearly shown to contain an active 16-hydroxylase Not only are 16-hydroxylated phenolic steroids found in the urine, but there are also significant amounts of C_{19} and C_{21} steroids with hydroxyl groups in position 16 *(169–174)* A number of these have the same Δ^5-3β-hydroxy structure as dehydroepiandrosterone and pregnenolone The frequency with which this structure occurs suggests that this spatial configuration fits the active center of the 16-hydroxylase better than the Δ^4-3-keto structure and the 3α-hydroxy metabolites thereof Probably the saturated compounds arise by reduction of the double bond after 16-hydroxylation. Thus far there is no clear information regarding the site of these changes.

More curious members of this group are three compounds having a 16-oxy group but no oxygen on C-17 These have been isolated from pregnant mares' urine, the same source from which equilin and equilenin were obtained At least in this species, which seems to have unique steroid metabolic systems, there appears to be a 16-hydroxylase which does not require a prior substitution on carbon-17 *(175, 176)*

Besides the various 16-oxysteroids a number of phenolic steroids have been isolated from urine hydrolyzates, the origin of which is unexplained Marrian's group have demonstrated 18-hydroxyestrone (LXXXIII) *(177)*, yet the only clear occurrence of an 18-hydroxylase is in the adrenals, where the ultimate product is aldosterone. Also two groups of workers have confirmed the presence of 2-methoxyestrone (LXXXV) as a metabolite of 17β-estradiol-16-C^{14} *(178, 179)*, a reaction which has not, thus far, been observed *in vitro*. There are, therefore, other metabolic reactions involving the steroids which have not yet been associated with enzymes in specific tissues

2. *General Considerations*

The general purpose of hormones is to regulate the rate of certain biochemical processes so that the animal can successfully maintain itself and reproduce its kind in spite of changes in the environment. It should be noticed that, with the possible exception of aldosterone, the purpose of the steroid hormones is *not* to maintain a constant internal environ-

ment against changes in the external milieu, but to cause definite changes in the internal metabolism which would be beneficial to the continued existence of the species For instance, the steroid hormones of the reproductive tract are essential to the marked local and general metabolic changes which must take place during the process of reproduction in the vertebrate, particularly the mammal The metabolic hormones of the adrenal cortex increase protein catabolism during stress, including fasting, so that essential hepatic processes, such as the maintenance of glucose levels, can be maintained or accelerated The response of the adrenal steroids to inflammation holds the inflammatory reaction in check, so that in destroying the abnormal causative factors it does not also overwhelm the normal tissues. To relate the output of these hormones to a need which requires variation in the internal environment rather than constancy, the vertebrates have developed a system whereby the production of all the steroid hormones except aldosterone is controlled by release of pituitary hormones, which in turn are partially under the control of the nervous system

The levels of circulating steroid hormones would not be related to immediate pituitary control, however, if the active molecules remained in the circulation for long periods of time, or the products of their metabolism could be reused in the biosynthetic chain Thus we find that the active steroids have relatively short half-lives in the extra-cellular fluids, and the ultimate products of hepatic metabolism, the major system of removal, are not intermediates in steroid hormone biosynthesis This rapid turnover is essential to the function which these hormones play in the organism

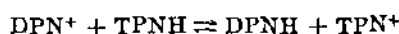
This does not mean, however, that small amounts of active molecules taken up by the cells at the site of hormonal action have the same short half-lives The major portion of these are probably attached to the larger molecules the action of which they affect But they exchange with the molecules free in the intracellular medium, which in turn exchange with those of the circulating extra-cellular media Thus the half-life of molecules involved at the active site is probably somewhat longer than the extra-cellular value The proportion of steroid hormone molecules which actually reach the active sites, however, would appear to be small; most of the output is involved in maintaining the proper concentration gradients

V. Mechanism of Hormonal Action

by the steroid hormones are varied, including protein synthesis, fat and carbohydrate metabolism, and electrolyte distribution. Moreover, some of these effects are quite localized The effect of a given hormone is not limited to one process, however· androgens cause protein anabolism in muscles as well as fructose synthesis in the prostate gland or seminal vesicle Thus mechanisms of action have been sought which might be common to a number of processes

Because of the nature of the steroid molecule, the first concepts were those of action of the hormone on the "permeability" of the cell, this word, of course, hiding a world of ignorance regarding how such effects were specific Some hormonal effects may be brought about by the attachment of the steroids to specific molecules located in the cell surface Mueller, however, showed that if a single dose of 17β-estradiol is given intravenously to ovariectomized rats and the uteri are removed at various intervals thereafter, a progressive increase in rate of incorporation of glycine-2-C^{14} into the protein of uterine slices is obtained *in vitro* which, when extrapolated, intercepts the control rate at the injection time. A similar immediate response could be demonstrated when serine aldolase activity was tested in uterine homogenates (*180*). Estradiol itself added *in vitro* had no effect, but the 2-hydroxy and 4-hydroxy derivatives had a significant estradiol-like influence, raising the question of possible conversion in the intact rat (*181*). These experiments indicated an action on amino acid activation which was not the result of changes in cell permeability, but did not distinguish between a direct influence on the activating enzyme and an effect on availability of some cofactor

The first clear-cut evidence of the direct activation of an enzyme by a steroid hormone is the recent work on an estrogen-dependent nucleotide transhydrogenase in placenta (*182, 183*). In the presence of very low concentrations of 17β-estradiol, estrone, or several other estrogens this enzyme will catalyze the reversible reaction.



$$DPN^+ + TPNH \rightleftharpoons DPNH + TPN^+$$

The assumption is that the steroid attaches to the apoenzyme as a prosthetic group and undergoes repeated oxidation and reduction

Since placenta also contains a 17β-estradiol dehydrogenase, Talalay on the basis of parallel activity over a considerable range of purification came to the conclusion that the two enzymes were the same (*184*) He then advanced the hypothesis that this might be a general mechanism of steroid hormone action, since the reduced or oxidized forms of one of the two nucleotides is involved at some point in the different processes affected by steroid hormones The various hydroxysteroid dehydro-

genases, using both TPN and DPN to some degree, could each act as DPN-TPN transhydrogenases It was shown, for instance, that hepatic 3α-dehydrogenase could function in this manner (*185*) As was pointed out earlier, however, this reaction would tend to run in only one direction since both the 5α- and 5β-androsterones are rapidly conjugated with glucuronic acid For transhydrogenase activity the steroid must undergo alternate oxidation and reduction

The concept has been attacked on several counts First, evidence has been introduced that the estradiol-sensitive transhydrogenase of placenta and the 17β-estradiol dehydrogenase are not the same enzyme (*186*) This would not invalidate the concept of the estrogen as a prosthetic group undergoing oxidation and reduction while attached to the transhydrogenase, however Attempts to find such steroid-sensitive transhydrogenases in other target tissues, such as the secondary sex glands of the male, have thus far been inconclusive Further, the actual relationship of the placental enzyme to a biological phenomenon connected with estrogen action has not yet been demonstrated Lastly, the rates of transhydrogenation are so low compared with the mitochondrial nucleotide transhydrogenases (*187*) that even the placental enzyme could only have a significant effect if its influence in the cell *in vivo* were localized

Hydrogen transport as a possible mechanism of estrogen action may occur by other than oxidation and reduction of an alcohol group Williams-Ashman *et al* (*188*) have shown that phenolic estrogens may function in this capacity in the presence of purified phenolases, the steroid molecule undergoing an initial hydroxylation to an ortho-diphenol derivative which then enzymically donates hydrogen to the nucleotide with the formation of an ortho-quinone The reduction of this to the diphenol by the hydrogen donor is probably nonenzymic Estrogens also transported hydrogen in the presence of peroxidases, probably through the formation of phenoxy radicals (*188*) Obviously, these particular mechanisms would apply only to phenols

While these experiments give no final answer, and may even be somewhat removed from the true mechanism of action, the effect of the steroid hormones may lie in the area of hydrogen or phosphate transport whereby the availability of certain cosubstrates used in more than one reaction is affected The influence in a given tissue would then be determined by the presence of both an enzyme in which the steroid can serve as a prosthetic group for the transfer, and the enzymes which can utilize the cosubstrates Another possibility is that the specific binding tendencies of the steroids for particular structural relationships in the proteins may block certain enzymes and thereby increase available substrate or cofactor for another system With the increased knowledge of such

mechanisms as protein synthesis, and better understanding of localization of function within the cell, the answer to the problem of mechanism of hormonal action, particularly in the case of the steroids, should be forthcoming in the near future.

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Carotenoids and Vitamin A

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I. Introduction

Carotene was crystallized in 1828, but it was not until 1907 that its percentage composition was finally settled by Willstätter and Mieg In 1919, Steenbock suggested a relationship between carotene and the fat-soluble A factor of McCollum and Davis, but twelve years of uncertainty were to pass before Karrer and his co-workers established the structures of β-carotene and vitamin A. The total synthesis of several common carotenoids was achieved in 1950.

Certain carotenoids are capable of serving as precursors of vitamin A, but in most respects their precise functions are not yet unequivocally discerned The experimental approach today places far less emphasis on the chemistry and much more on the physical mechanism, and the questions asked are in some respects more sophisticated—if carotenoids participate in photosynthesis, by what process do they transfer the absorbed energy to chlorophyll *a*—and in answering—"by internal conversion"—we have to consider the efficiency to determine the plausibility. Do we find an unpaired electron (as in a free radical)—if so, can we apply the method of electron-spin resonance, and how do we interpret the result? Failure to take a look at these concepts means that we ignore a large and rapidly growing literature, much of it having a direct bearing on the biochemical role of the carotenoids

There are very few references to carotenoids or vitamin A in the first edition of this monograph One reason is undoubtedly because carotenoids, as such, have no major role in animal biochemistry, except insofar as we are dependent upon carotenoids from land plants or phytoplankton for vitamin A There is no known case among the higher animal phyla of *any synthesis of carotenoid de novo Such carotenoids as may* be found in animals are of plant origin to begin with, whether or not they have undergone some modification in the animal

II. Scope

This chapter will deal first with the structural chemistry of the carotenoids, as a special class of naturally occurring polyenes, and their relation to other classes Here the writer will avoid, so far as possible, covering information available in well-known monographs (*1*, *2*). The properties of the polyenes will be considered next, and *special emphasis* will be placed on their ability to form complexes of varying stability in the presence of a proton donor or an electron acceptor. The reason for this emphasis is that it is the major key, in present thinking, to the probable role of these compounds In fact, for rhodopsin the rod pigment, as will be noted later, the suggested chromophore *is a protonated* Schiff base

This will lead naturally to a consideration of the role of carotenoids in energy transfer to chlorophyll *a*, and their possible role in electron-transfer mechanisms

Carotenoids in phototropism and phototaxis will be reviewed The coleoptile of a germinating wheat seedling bends toward the light and two major theories have been offered One suggests that the photoreceptor is carotenoid, the other that it is riboflavin A study of this controversy

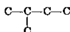
is well worth while to any experimenter dealing with action spectra, because in the case cited, the well-defined two-banded spectrum is almost certainly that of a carotenoid, yet the interpretation is open to doubt

This discussion will be followed by a section on functions in animals, by evidence showing transformations from more saturated to less saturated polyenes, and finally by a detailed discussion of the biosynthesis of this extremely interesting class of compounds Here the ramifications are numerous One becomes involved in various phases of amino acid metabolism, lipids, sterols, the fate of branched chain acids, recycling of fragments with the acetoacetate pool and so forth

III. Carotenoids as a Class of Naturally Occurring Compounds

Pedagogically, carotenoids constitute a special class of polyenes, in which a structural relationship to isoprene can be discerned They are therefore members of the wider group, isoprenoid compounds Since the majority of carotenoids have 40 carbon atoms, they are sometimes placed in a subgroup with terpenoids and labeled tetraterpenoids, which is not a particularly useful designation

Classifications are aids in bringing more information within the grasp of the individual mind The isoprenoid skeleton is seen as a



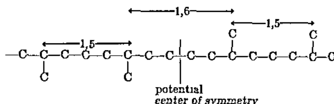
repeating unit in rubber, in terpenes, in phytol, in the carotenoids, and in a large number of related compounds and makes such a classification well-nigh inevitable in view of this purely structural relationship

There are however times when a premature arranging of facts hinders our thinking and delays an orderly development of the subject This often seems to be the case when we consider the biochemistry of isoprenoid compounds, as distinct from their formal structural chemistry

Higher plants synthesize both sterols and carotenoids, and this is also true of many fungi and yeasts A few bacteria synthesize carotenoids, but not sterols Animals synthesize sterols but not carotenoids, although they can make squalene

By definition, a carotenoid is colored, as a result of a sufficient number of conjugated double bonds, it is structurally related to isoprene, so that it has a series of methyl side chains These are separated 1,5 except for the two nearest the center which are separated 1,6 Consequently the

typical carotenoid has a potential center of symmetry:

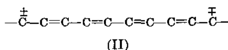


The characteristic structure may be represented as an extended chain lying in the plane of the paper, shown in its all-*trans* form [structure (I)]



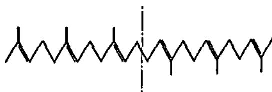
(I)

Zechmeister's contributions (*3*) laid the basis for an understanding of the spectral changes as the various double bonds undergo *trans-cis* rotations, and the freedom of the π-electron to resonate along the chain which can be represented for convenience by structures such as:



(II)

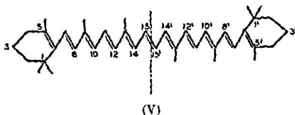
Squalene, $C_{30}H_{50}$ (III) has six double bonds, none in conjugation, while vitamin A, $C_{20}H_{29}OH$ (IV) β-carotene, $C_{40}H_{56}$ (V), and lycopene, $C_{40}H_{56}$ (VI), have five, eleven, and eleven, respectively, in conjugation.



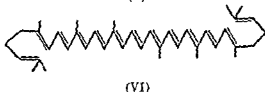
(III)



(IV)



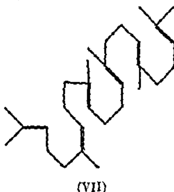
(V)



(VI)

The cyclo-hexenyl rings in β-carotene and vitamin A are puckered, and the double bonds in each ring have lost somewhat in double-bond character. Spectroscopic evidence places each at one-half the value of a double bond in an open chain. Lycopene with 13 double bonds has 11 in conjugation, and β-carotene with 11, two of which are in rings, has therefore the equivalent of 10. (α-Carotene[1] by similar reasoning has the equivalent of 9.5, and γ-carotene 10.5 effective bonds in conjugation.)

Squalene is represented above as an extended chain solely to show the structural similarity to all-*trans* lycopene. It has more often been written in an arrangement



(VII)

resembling the formal representation of cholesterol, but such a pictorial representation is incorrect, as all-*trans* squalene is symmetrical.

[1] The reader is referred to the above-mentioned monographs (*1*, *2*) for numerous structures mentioned here *en passant*.

These representations do, however, illustrate one characteristic of terpenes, namely, that they are able to cyclize readily. Squalene with no double bonds in conjugation is in all respects but one a typical acyclic terpene In terpenes, sesqui-terpenes, and diterpenes, the methyl side chains are nearly always separated 1,5, whereas the central methyls in squalene are 1,6, in which respect it resembles the carotenoids The transformation of squalene to cholesterol, via lanosterol is completely outside the scope of this chapter, but similarities between squalene and phytoene, $C_{40}H_{64}$, an octahydro-lycopene, are not The latter is also symmetrical It has nine double bonds, six of which are isolated, but the three central ones are in conjugation (*4*)

Conventional methods for identifying squalene include one or more of the following the formation of a crystalline hexahydrochloride, infrared spectrum, and halogen uptake The hexahydrochloride can be resolved into three isomers, and melting points of preparations vary 10 to 15° or more, depending possibly upon isomerization during the reaction, dry HCl being bubbled through an ether solution The presence of squalene in yeast (*5*) and olive oil (*5a,b*) is established, but evidence for its distribution in low concentration in other plants is not completely unequivocal where phytoene and phytofluene are present

IV. Properties of Polyenes

We must seek the biochemical role of these compounds in terms of their structure They are highly unsaturated, so that formation of epoxides, furanoxides, and conceivably peroxides is to be expected. They have also a potential role as hydrogen acceptors.

Because of their conjugated double bond system they are colored and show strong absorption in the blue They are present in many tissues in sufficiently high concentration that a very high percentage of the incident light at certain wavelengths is in fact absorbed by them. The use of this energy in photochemical reactions, particularly in photosynthesis, must therefore be considered, also harmful misuse The latter may be prevented either by subsequent active participation of the carotenoid, in an electron-transfer mechanism, or, more passively, as in one theory of phototropic behavior, by acting as a light filter and dissipating the energy as heat

Finally an important property conferred by the polyene structure is the ability to form a highly colored carbonium ion complex in the presence of a proton donor or an electron acceptor. This property is common to all conjugated dienes. Thus the deep blue coloration of vitamin A in the presence of concentrated sulfuric acid—(more recently with antimony

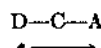
trichloride)—was a test for cod liver oil before vitamins were known The green and blue complexes with proteins formed by carotenoids in crustaceans, in many locust wing and body pigments, and indeed visual purple (rhodopsin) formed with the protein scotopsin by a vitamin A derivative must fall in the same general category The precise structure of such compounds may be very difficult to determine Strongly negative ketonic oxygens in the carotenoids of crustaceans might well enhance the stability of a nearby carbonium ion, and differences in stabilities of the various complexes may thus be accounted for

The possibility of structures such as vitamin A forming a cation by donating an electron to a suitable acceptor has also been suggested [Meunier and Vinet, cited by Fukami *et al* (*6*)] The introduction of the charge into the conjugated chain increases the resonance with a resultant shift of the absorption maximum to longer wavelengths This property is characteristic of Mulliken's charge-transfer complexes (*7*) If we represent a carotenoid, an electron acceptor and a donor respectively by C, A, and D, the complex may be written



$$C^+—A^-$$

We then consider a further stage in which electrons are continuously transferred from D to A via C



$$\underset{e \longrightarrow}{D—C—A}$$

This forms the basis for Platt's recent discussion (*8*) and is repeatedly considered, for example, by Calvin and co-workers (*9*). A may be a quinone, semi-quinone, or other electron acceptor spatially removed by design or necessity from D Without C, the charge-transfer complex would not come into being

The biochemical roles are obviously dependent upon the inherent potentialities of such structures as these

V. Carotenoid Functions

A Transfer of Energy to Chlorophyll *a*

Until recently it has been accepted that four quanta of visible light are required for the reduction of one mole of carbon dioxide in the photosynthetic reaction Because it was impossible to conceive of a chlorophyll molecule absorbing successively one, two, three, and then four quanta and transferring the accumulated light energy into chemical bond energy, a photosynthetic unit was then postulated To account for the effectiveness of light energy in photosynthesis in spectral regions where chlorophyll absorption is low, a mechanism was sought whereby the energy

could be successively transferred from one pigment to another in a "bucket brigade" until it reached chlorophyll *a*, the primary photosynthetic pigment [2] The other pigments, including chlorophyll *b* and the carotenoids, are termed "accessory pigments" and they participate in the process, as shown by the fact that only the fluorescence of chlorophyll *a* is excited, regardless of the wavelength of the absorbed light.

Unfortunately, the evidence for participation of accessory pigments is strongest for those of noncarotenoid nature, and therefore not germane to this review, apart from fucoxanthin, $C_{40}H_{56}O_6$, the occurrence of which is restricted to the brown seaweeds and diatoms Any general claim for carotenoids as agents in energy transfer must include proof that the function exists in the photosynthetic mechanism of higher plants, or at least in that of the green algae whose pigment complex is essentially the same At the risk of prejudging the evidence, we shall cite first the conclusions of Blinks (*10a,b*), who contrasted action and absorption spectra of numerous organisms He found some ineffective absorption in the regions where carotenoids absorbed a high proportion of the light, yet photosynthesis fell by a factor of only 20% "This action spectrum, as well as many others, forces one to conclude that most of the pigments, *including carotenoids*, are photosynthetically effective in green plants"

While this view has widespread acceptance, the reader should weigh a recent statement by Emerson and Chalmers (*11*) that "The extent to which light energy absorbed by carotenoids other than fucoxanthol (fucoxanthin) can be contributed to photosynthesis is uncertain so for the present we are not including them among the accessory pigments"

The methods for deducing participation by accessory pigments include manometric techniques from which quantum yields were calculated [Emerson and Lewis (*12, 13*)], oxygen by polarographic measurement [Haxo and Blinks (*14*)], and fluorescence action spectra (*15–19*). With respect to the fluorescence, three statements may be made.

1. Light quanta absorbed by fucoxanthin excite chlorophyll *a* fluorescence as strongly as do quanta absorbed by chlorophyll *a* itself

2. Chlorophyll *b* transfers its energy to chlorophyll *a* with high efficiency Only the latter fluoresces, as shown by the action spectrum

3. In the photosynthetic bacterium, *Chromatium*, there is transfer of light energy from the carotenoids to bacteriochlorophyll B890 (so named from an absorption maximum at this wavelength This pigment-protein conjugate is the only one of three such conjugates which fluoresces). Transfer also occurs in *Rhodospirillum* from carotenoids

The transfer in the cases listed in item three above has an efficiency of the order of 35 to 50% The argument is as follows. if transfer were

[2] Bacteriochlorophyll in purple bacteria, bacterioviridin in green bacteria

complete, the fluorescence action spectrum would correspond with the total absorption by the bacteria; if there were no transfer, it would correspond with that ascribable to bacteriochlorophyll, with very limited fluorescence excitation between 450 and 550 mμ where the carotenoids absorb strongly

We must now examine Mulliken's complexes (7) in more detail As Lumry *et al.* (20) remark, these weak, generally nonspecific intermolecular interactions can be very important for living organisms Briefly the theory involves resonance between (A, B) or (A^-—B^+) where A is the acceptor atom, molecule, or ion, and B the donor The molecular compounds A.B range from loose complexes to stable compounds A special class of intense electronic absorption spectra is predicted for A B which is nonexistent for either partner alone These are called charge-transfer spectra, and the forces leading to complex formation are termed charge-transfer forces What is of special interest here is the deduction that they have characteristic and "specific orientational properties of possible importance for the manner of packing of molecules in liquids in molecular crystals, in heterogeneous systems and in biological systems." We have only to mention the highly organized chloroplast structure to appreciate the applicability of the theory in photosynthesis Whether these interactions are considered in terms of the tendency of a conjugated polyene to form carbonium ion complexes, governed in part by the proton donor (e g , in an extreme case, the polyene reaction with concentrated sulfuric acid), the complexes formed in living organisms set the stage for photochemical and subsequent chemical interactions

Three possible modes of transfer of energy are examined by Arnold and Oppenheimer (21).

1. Collisions of the second kind, involving transfer from one molecule to another on collision This would predominate when the two systems are separated (during the transfer) by distances of the order of atomic dimensions

2 Emission of radiation from the one and its reabsorption by the other. This depends critically on the existence of resonance between the two, and will operate when the distances are of the order of several wavelengths

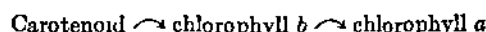
3 Internal conversion, a resonance transfer of energy from one oscillator to the other, and lying within the quasi static rather than the wave zone field of case 2.

The distinction is not absolute, but in the case of dye-chlorophyll transfers, only the last-mentioned can be important If, as is experimentally found in *Chroococcus*, 90% of the light is transferred, such efficiency cannot be expected by random collisions In this organism, by a

process analogous to internal conversion in radioactivity, but with linear dimensions and wavelengths four orders of magnitude larger there are transferred about 100 quanta from the accessory pigment to chlorophyll *a* for every quantum emitted as fluorescence by the accessory pigment in the *Chroococcus* cell Unfortunately in this illustration, the accessory pigment is phycocyanin, not a carotenoid, and in the well-authenticated case for equally efficient energy transfer with fucoxanthin in diatoms, we have no information on fluorescence of the fucoxanthin-protein conjugate.

Arnold and Oppenheimer estimated the fate of the absorbed energy for phycocyanin to be fluorescence, 1%, heat losses, 4%; internal conversion, 95% In general the transfer for carotenoids is not nearly so efficient Blinks (*10a,b*) showed there is a substantial amount of inactive (photosynthetically ineffective) absorption by carotenoids In certain algae particularly those exposed to xerophytic environments, in salt brines, or under epiphytic conditions, carotene may be found in oil droplets, topographically removed from the photosynthetic sites

The general concept which has emerged suggests a bucket brigade of pigments, which, for spectral regions where chlorophyll *a* absorbs only a small fraction of the energy may be represented by·



The foregoing would represent the situation for a green alga or a higher plant and would be modified according to the presence or absence of any particular accessory pigment To this we may add recent conclusions by Emerson (*22*) concerned with quantum yield measurements, that "all absorptions of shorter wavelengths, by whatever pigment, have a good chance of leading to the first excited singlet state of chlorophyll *a* . If the primary absorption were by some carotenoid of green algae, the path might be through chlorophyll *b* to *a*."

However, for this purpose alone, the typical chloroplast has an unnecessarily large and varied assortment of carotenoids, as shown by Strain (*23*) Furthermore, except as an adaptation to meet competitive conditions, carotenoids cannot be obligatory components in the primary photosynthetic step, in an energy-transferring system to chlorophyll *a* For this, two reasons may be adduced the chlorophyll *a* may absorb the light directly, carotenoid-free mutants of photosynthetic bacteria can live autotrophically in the light in the absence of oxygen. This last-mentioned finding, by Griffiths *et al* (*24*), leads to consideration of a second carotenoid function, discussed in the next section.

Franck's latest proposal (*25*) would "avoid some necessity for a chemical bucket brigade up the energy ladder [Rosenberg (*26*)] If we suppose that the carbon involved in the photochemical step is more

reduced than CO_2, we no longer need a minimum of four quanta, and other explanations become possible The splitting of water could be achieved with two quanta, and the reduced radical (H) might react with phosphoglyceric acid By dismutation, two of these partly reduced molecules would yield triose and the original acid The peroxide radical would yield ultimately peroxide and oxygen

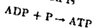
Attention must be drawn to a detailed exposition of the findings of Arnon and his co-workers over the past five years, and published recently *(26a)* Both green plants and photosynthetic bacteria have in common a mechanism for cyclic photophosphorylation of ADP to ATP as a result of the expulsion of a high energy electron from chlorophyll on absorbing a quantum of light Green plants have in addition, a second cyclic and one noncyclic pathway of photophosphorylation The phosphorylations are kept going in both cases via the cytochromes, but the details of the electron transport mechanism are different

In the cyclic paths, the electron may be restored to the chlorophyll by one of two means

1 Via a vitamin K-like quinone to a terminal cytochrome component and then directly to the chlorophyll The oxidation of the cytochrome is coupled with the phosphorylation This is independent of chloride

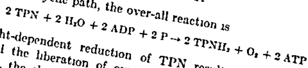
2 Via riboflavin phosphate (FMN type) and a cytochrome chain This process is chloride-dependent

In both cases, the over-all reaction is



$$ADP + P \rightarrow ATP$$

In the noncyclic path, the over-all reaction is



$$2\ TPN + 2\ H_2O + 2\ ADP + 2\ P \rightarrow 2\ TPNH_2 + O_2 + 2\ ATP$$

his light-dependent reduction of TPN results in the formation of TP and the liberation of oxygen To keep the chlorophyll system erating, the electrons removed (not returned as in the cyclic paths) st be replaced so that the chlorophyll can return to its normal state on supplies the electrons from water dissociated into H^+ and OH^-. OH^- supplies the electrons and yields oxygen, while the protons are ved as TPNH The photolysis of water to yield H and OH radicals is eliminated

or carbon assimilation in bacterial photosynthesis, an external gen donor is needed This can be dispensed with in the green plant is handled by the light-dependent reduction of TPN Thus the characteristic feature of photosynthesis by green plants, namely lution of oxygen, becomes an incidental consequence of the path to produce the reductant

It may be noted that Bergeron (*26b*) has been able to vary the efficiency with which quanta absorbed by the carotenoids in *Chromatium* are used in photophosphorylation.

Changes in the spectral absorption of the bacteriochlorophyll-carotenoid complex between 800 and 900 mμ are associated with the effectiveness of the energy transfer. Carotenoid deficiency and also uncoupling of the complex are shown by an increase at the 800 mμ peak, and decreases for the peaks at 850 and 890 mμ

It will be seen that the experimental evidence for the role of carotenoids in energy transfer to chlorophyll *a* is based on a comparison of absorption spectra and some response of the organism. Measurements of absorption spectra of living cells are still not easily perfected. Latimer and Rabinowitch (*27*) found anomalous higher scatter on the long wavelength side of an absorption band, and lower on the shorter side. The *Chlorella* cu [illegible]
carotenoid
spectra, mu
We cannot, however, avoid the conclusion that the evidence preponderantly favors a role for carotenoid in the primary photosynthetic step, and that in a complex of the type characterized by Mulliken, the transfer of energy proceeds by internal conversion.

B. Carotenoid Functions in Electron Transfer

Bassham and Calvin (*9*) viewed the problem somewhat differently. They concur that the energy is either absorbed directly by chlorophyll *a* or transferred to it The fact that light absorbed around 480 mμ produces a lower quantum yield indicates either inefficient transfer to chlorophyll *a*, or that the energy is in part transferred to other reactions In this event, there should be some effect on subsequent chemical steps. Although they cite some Russian work showing an enhanced protein: carbohydrate ratio for blue versus red light, they suggest that such effects are due to changes in relative rates of transformations of intermediates into other substances A photocatalytic effect is similarly ascribed as an explanation for Warburg's manometrically measured quantum yield with red or green light plus catalytic amounts of blue. They suggest that a charge separation is induced by a quantum absorbed by chlorophyll, and this separation may be made irreversible by an electron acceptor at one end, isolated from a donor at the other by means of a semiconductor, such as lipid material, with occasional conductor molecules, the carotenoids

This view is later developed by Tollin *et al.* (*28*) "that aggregates of chlorophyll molecules in the chloroplasts might give rise to conduction

bands in which photoproduced electrons and holes could migrate" We see here how Platt (*8*) could develop the concept of a "trimolecular charge-transfer complex," created by an electron donor at one end of the carotene molecule, and an acceptor at the other

The theory discussed by Platt is based on the fact that "a conjugated chain of *n* atoms has a maximum wavelength of absorption only when it can be written with two extreme resonance structures that are equivalent" For carotene, *n* would be 22, and the "isoenergetic wavelength" [illegible]

imply interactions with acids and bases of the Lewis type, but apparently this was first explicitly stated by Körosy [a] Warburg *et al* (*30*, *31*) also suggest that carotenoids participate in electron transfer between the photochemical reaction and reduction of CO_2 as an explanation of the blue light effect

We must now consider an important contribution from Stanier's laboratory, referred to in the previous section (*24*) A universal function for carotenoids in photosynthesis is postulated, namely, the protection of the cell from photodynamic destruction by chlorophyll This is based on a comparative study of *Rhodopseudomonas* mutants One, blue-green in color, was devoid of carotenoid, except for the colorless phytoene, and could exist as a phototroph only when oxygen was excluded In the authors' words "If protection from photosensitization is a general carotenoid function, then the carotenoid-free condition can be compatible only with photosynthetic growth in bacteria since these are the only phototrophs that can be grown in the light in the complete absence of oxygen" Calvin (*32*) has attempted to formulate a molecular basis for protection against this photo-oxidative destruction He argues that if carotene has the ability to remove 25–40 kcal excitation energy in some useful way prior to its use in activating some oxidation reaction by molecular oxygen, then the protective action of the carotene is incidental. He points out that photosensitized oxidations by molecular oxygen and dyestuffs such as chlorophyll are well known One mechanism for such an oxidation involves transition of the first excited singlet state into a longer-lived triplet state The latter could give rise to a reactive peroxide which would transfer its oxygen to a suitable reductant, leaving the sensitizer to return to its original ground state. If we assume that *carotene* can play a part in the transport of energy from the longer-lived triplet state, then, in the absence of carotenoid, there would be a greater proba-

[a] The article mentioned by Platt is not immediately available to the writer A preliminary earlier note is to be found in *Experientia* (*29*)

bility for active peroxide formation in the presence of oxygen. Calvin then cites the evidence of absorption spectra Peaks in the wild form of *Rhodopseudomonas* are found at 805, 855, and 880 mμ, and in the mutant, a symmetrical band at 875 mμ with shoulders around 770 and 812 mμ Specifically he suggests that the missing bands are associated with a pi-complex between the chlorophyll and carotenoid, which would indicate a close coupling between the electronic systems of the two molecules and provide a very effective pathway for energy transfer

However data from electron spin resonance measurements lead Ingram (*33*) to make the statement that an excited triplet state is improbable, and a brief consideration of the technique may be useful.

The magnetism of elementary particles is associated with angular momentum. If, as a result of the absorption of light, we create a free radical, there is in consequence an unpaired electron whose spin can be measured In zero-magnetic field, the spins will be randomized, and consequently also the magnetic moments With a field of intensity H, the electrons will align themselves with spins and moments parallel or antiparallel to the applied field, and two groups will exist, with $\frac{1}{2}\Delta E$ less and $\frac{1}{2}\Delta E$ greater than the zero-field value, commonly written $-\frac{1}{2}g\beta H$ and $+\frac{1}{2}g\beta H$, where the g-value or spectroscopic-splitting factor "is a measure of the contribution of the spin and orbital motion of the electron to its total angular momentum" [Ingram (*33*)], to which a value of 2 000 is usually assigned H is the field intensity in gauss, and β the Bohr magneton, 0 9274 × 10^{-20} erg per gauss The energy difference is in the *microwave region, around 0 3 to 20 cm As the magnetic field is varied,* an absorption band will be detected, governed by the expression

$$h\nu = g\beta H$$

To induce free radical formation, the sample (usually a chloroplast preparation) is irradiated with light around 680 mμ, the absorption maximum for chlorophyll *a in vivo* With a microwave beam of wavelength around 3 cm a signal[4] is detected as H approaches the value required by the above expression More data are required before carotenoid participation in the process can be clarified.

Some years ago it was shown that if two solutions of chlorophyll are *exposed to red light in the presence of oxygen,* and if to one of the solutions a small amount of carotene has been added, the carotene has a protective effect on the photo-oxidation of the chlorophyll (*34, 35*) This effect occurs at wavelengths where carotene shows no absorption

[4] The signal, so-called, is in reality a plot of the absorption on the recording device, as a function of H

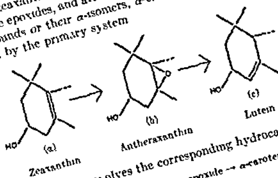
Furthermore the carotene-chlorophyll spectra are strictly additive, so that no complex formation is likely Since it would be an unrewarding task to try to show the carotenoid-free condition to be compatible with growth in higher plants, a more detailed study of the above-mentioned reaction might provide clues as to the mechanism whereby Stanier's postulate can be given a molecular basis

C. Carotenoids and Peroxide Formation

A role for the carotenoid in peroxide formation has been suggested more than once It was envisaged speculatively by Lumry *et al* (20) in reevaluating estimates of heat loss for oxygen production in photosynthesis, and an experimental test had been attempted earlier by Dorough and Calvin (36), who explored the possibility of transfer of oxygen along the conjugated chain The two oxygens from a diepoxide meeting in the center would form a peroxide On decomposition, oxygen would be evolved and the original carotenoid regenerated The difficulty in an experimental test with water containing O^{18} lies in the small quantity of carotenoid involved Furthermore, the oxygen might come from molecular oxygen, as is the case for lanosterol from squalene, and this would add to the experimental difficulty if a green phototrophic organism were used

D. Carotenoid Functions in Oxygen Transport

An interesting hypothesis that carotenoids serve in oxygen transport is advanced by Cholnoky *et al* (37) The hypothesis is that in the first instance, carotenoids with the β-ionone configuration are formed, i e, β-carotene and zeaxanthin which is 3,3′ dihydroxy β-carotene These are converted to the epoxides, and after reduction they are reconverted to the original compounds or their α-isomers, α-carotene and lutein The main load is carried by the primary system



The secondary system involves the corresponding hydrocarbons

β-Carotene → β-carotene monoepoxide → α-carotene

Hence in leaves containing chlorophyll there is little or no zeaxanthin and a great deal of lutein The predominance of β-carotene over α-carotene is presumably due either to limited participation of the secondary system, or to preferential reversion of the epoxide to β-carotene Epoxides and lutein continue to be prominent in the unripe fruit but disappear in the ripe fruit. The epoxides are converted to ketones[5] and no longer function in oxygen transport, and zeaxanthin appears. The authors emphasize that the role of carotenoids in photosynthesis can only be understood by considering all the carotenoids and their relative changes during the life cycle of the plant The first objection, that in plants such as ivy, or a redwood, where young and old leaves are readily distinguishable (38), there should be an increase in the percentage of α-carotene with age, can be met by the argument that there is a constant turnover, and one may be simply measuring the conditions of a steady state A second and more serious objection is that as chlorophyll disappears, a rigid control imposed by the necessity to conduct photosynthesis in a highly organized system is relaxed, and one cannot predict *a priori* what the organism will do. In an apple, for example, it synthesizes negligible quantities of carotenoid, in the tomato it makes lycopene Consequently one may properly argue that data on the ripening fruit are—in general—irrelevant to the hypothesis.

We are left therefore with the observation that leaves of higher plants contain predominantly lutein and β-carotene together with small quantities of their epoxy derivatives It is hardly open to question that the latter are derived from the former, but the unanswered question is how and when this takes place—during illumination as part of the photosynthetic process or in the dark The oxygen might come from water, molecular oxygen, or a quinone

E. The Hill Reaction

This reaction, named after its discoverer (39), involves formation of a reductant in the primary photochemical step It may be easily measured by reduction in the light of the dye 2,6-dichlorphenol indophenol. Lynch and French (40) freeze-dried chloroplast grana, eliminated nearly all the activity by petroleum ether extraction, restored it by adding back the extract, and obtained partial restoration to around two-thirds of the original, with pure β-carotene Subsequent work (41, 42) makes it possible that the effect is nonspecific, that vitamin K may be involved, and that carotene merely prevents a photo-oxidation when menadione is used alone. However, participation of carotenoid in the Hill reaction in the normal untouched chloroplast is not necessarily excluded.

[5] This is true of the red pepper, *Capsicum* sp , but it is not a general phenomenon

F. Carotenoid Functions in Phototropism and in Phototaxis

When the cotyledon of a monocotyledonous plant, usually wheat or oat, is illuminated unilaterally it bends toward the light This tropism is brought about by a difference in auxin concentration. The action spectrum is characteristically the two- (and even three-) banded spectrum of a carotenoid

According to one theory (*43*, *44*), the carotenoid has a passive role, acting as a light filter, protecting the auxin on the posterior side from destruction when the tip is illuminated unilaterally Galston (*45*) showed that riboflavin was a photosensitizing agent capable of catalyzing auxin (indoleacetic acid) destruction, and the reasoning is as follows, that the carotenoid in the oat coleoptile is concentrated in the tip, whereas the riboflavin is more uniformly distributed Consequently the action spectrum is that of the carotenoid, and there is an approximate coincidence of high phototropic sensitivity with highest carotenoid concentration in the tip However, the *in vivo* inactivation of auxin by riboflavin (*46*) and its extension to include flavoprotein (*47*) has been challenged by Mer (*48*), on the basis of the reference standard chosen to determine the phototropic curvature

The second theory interprets the data to mean that a carotenoid is the active photoreceptor According to the reasoning of Shropshire and Withrow (*49*), the phototropic curvature caused by unilateral illumination is produced by attenuation of the incident light as it is transmitted through the coleoptile. The attenuation, they reason, is due to self-screening by the photoreceptor, A_S; neutral self-scattering, A_N, foreign screening by inert pigments, A_F The total absorbance of the coleoptile, A_T, is the sum of the above. The absorption creates a photoproduct which determines cell elongation, and hence the curvature The quantity of photoproduct is determined by the quantity of energy E_1, and the absorption coefficient of the receptor, β Thus the magnitude of the growth difference is a function of A_T, β and E_1 Shropshire and Withrow then derive a general equation describing response θ

$$\theta = kA_T(\log E_1 + \log \beta)$$

To obtain the photoreceptor action spectrum, they set $\theta = 0$, to reduce the expression to

$$1/E_1 = \beta$$

Thus the action spectrum for the threshold response corresponds to the photoreceptor absorption spectrum, while for larger responses, the spectrum is multiplied by the factor A_T

Finally, to summarize their data and conclusions, the active photoreceptor is probably carotenoid, with absorption maxima at 410 to 415,

440 to 445, and 470 to 475 mμ A *fourth peak at 370 mμ is* tentatively ascribed to an inactive secondary pigment.

Phototaxis in flagellates is of two types, designated by the prefix topo-, or phobo-. The former is related to the direction of illumination, and the response may be positive or negative The latter is independent of direction, and depends according to Reinert (*44*) on "shock reactions" induced by intensity changes, and the organism can be made to stay within or to leave an area under illumination Again the response can be positive or negative. Once more carotenoids have been implicated since the blue-green region of the spectrum gives high phototactic responses, but from a careful analysis of the data, Reinert concludes this to be untenable, the nature of the pigments being unknown, apparently neither flavin (*50*) nor carotenoid (*51*).

G Carotenoid Functions in Animals

1 *General*

The ingestion of abnormally large quantities of carotenoids, whether from carrots, peppers, or some other rich source by man may cause the skin to appear yellow or even more strongly colored. Extreme cases exist in fish such as the Garibaldi or marine goldfish (*Hypsypops*), and salmon with its carotenoid-colored muscle tissue, and in birds such as the flamingo In all cases known to the writer where vertebrates are kept on a carotenoid-free diet, but supplied with vitamin A, health is maintained and the coloring fades

Individual Garibaldi captive for two to three years lost from 81 to 88% of their skin carotenoid, according to Fox (*52*) Ivory-white yolks of otherwise normal eggs were produced by hens on a carotenoid-free diet, by Palmer and Kempster (*53*) in an early test of Steenbock's theory and later, by Almquist *et al.* (*54*) even on a diet supplemented by carrots (low in xanthophylls but high in carotene) Recently Dadd (*55*) has developed a semi-synthetic diet for the desert locust. The absence of carotene suppressed the yellow color due to carotenoid pigments normally present in the integument Initial trials with vitamin A acetate gave satisfactory growth and survival, which is somewhat difficult to interpret, as vitamin A is apparently absent from the tissues. Dadd suggests possibly a derivative common to both carotene and vitamin A is the growth factor in question

While, therefore, pigmentation may influence phototactic responses, or, as an ecological factor even provide *protective coloration,* no known general biochemical function can be ascribed to carotenoids in invertebrate or vertebrate biochemistry with the important exception of those capable of acting as a provitamin A.

2. *Carotenoids as Provitamins*

The requirement for provitamin activity is that the terminal ring shall have an unsubstituted β-ionone ring configuration A possible exception is that of astaxanthin which may be a growth factor for shrimp. The main site of conversion, originally thought to be in the liver was shown by Mattson, Mehl, and Deuel (*56*) and in Morton's laboratory (*57*) to be the intestinal wall It has been suggested by Glover and Redfearn (*58*) that the C_{40} carotenoid molecule undergoes stepwise degradation which may commence at either end This means that the relative potencies of the carotenoids would remain unchanged, except with respect to vitamin A itself Thus, on a molar basis, β-carotene would be equivalent to the vitamin, and not have twice the potency which would be theoretically possible if it underwent central scission The mechanism has been modified by Fazakerley and Glover (*59*), who found that β-8′, β-10′ and β-12′-apo-carotenals had provitamin A activity for the rat [An 8′-apo-carotene means that scission of the carotene has occurred at carbon 8′, see Formula (V)] Two possibilities were considered. These compounds might undergo β-oxidation until the C_{20} of vitamin A was reached, or two C_5 units could be split from β-8′-apo-carotenoic acid and one C_5 from the β-12′-apo-acid

They therefore compared the activities of these C_{22} acids

1 β-14′-apo-carotenoic, retinylideneacetic
2 15-hydroxyretinylacetic

with the C_{25} acids:

3 β-12′-apo-carotenoic, retinylidenetiglic
4. γ-15-hydroxyretinyltiglic

If 3 and 4 were degraded by β-oxidation, 1 and 2 should appear, but this was not the case They found 4 versus 7–21% for the vitamin activity in the livers for C_{22} and C_{25} acids respectively. They conclude that a different enzyme system is involved handling the C_5 unit, and suggest tiglyl-CoA as a possibility in view of the work of Robinson *et al* (*60*) Tiglyl-CoA is converted by crotonase to α-methyl-β-hydroxybutyrate, and α-methylacetoacetate-CoA to acetyl-CoA and propionyl-CoA

Relatively little work has been done as yet on the fate of labeled carotene in the body. Fishwick and Glover (*61*) report that 2% of absorbed uniformly labeled β-carotene is eliminated as CO_2

VI. Vitamin A

A General

Details of absorption, storage, potency of the various *cis*-isomers, physiological responses, clinical manifestations, and pathology will not

be discussed here For these, the reader is referred to Moore's monograph (62), and on biological potency also to Ames (63).

Vitamin A is a growth factor for the young of mammals, birds, and doubtless of *many of the lower orders* *Locusts, for example*, require either vitamin A or carotene as already noted. Whether some insects can be raised on a completely vitamin A- or carotenoid-free diet may be questioned (The flour beetle and clothes *moth are examples normally cited* of such cases)

The condition known as xerophthalmia is one form of keratinization of epithelial cells, which develop a tendency to stratify. This may apply to all mucous membranes, and to the skin itself Moore suggests that the term anti-keratinizing vitamin would be appropriate.

As noted, carotene is converted to vitamin A in the intestinal wall. It is esterified and stored chiefly in the liver, where it represents around 95% of the total body supply. The remaining 5% is found in kidney, lungs, adrenals, blood (as the free alcohol), and retina. The retinal content is about 0 1% of the total

In the case of certain crustaceans, it is found almost exclusively in the eye, and in many insects, not at all, even though it be a growth factor. *In only one case, rod vision, has its function been adequately explained* at a biochemical level.

B. Vitamin A and Vision

Rhodopsin, porphyropsin, and iodopsin are among the retinal pigments which have been characterized They consist of retinene (retinene$_2$ from vitamin A_2 in the case of porphyropsin in fresh water fish) and a specific protein, and each conjugate has a characteristic maximum in the visible part of the spectrum. Iodopsin in chicken retina has a maximum at 565 mμ and is bleached by red light [Wald (64)]. The maximum for porphyropsin is at 522 mμ

Of greatest interest are the pigments involved in mammalian vision. Retinene, or more precisely neo-b-retinene (11-*cis* in Karrer's numbering, *4-cis according to the Geneva convention*[a]) combined with scotopsin, the rod protein has its maximum displaced from 384 mμ in digitonin solution to 500 mμ. On combination with the cone protein, photopsin, the maximum is found at 562 mμ (6).

Proceedings of a conference on photoreception were recently published. The following comments pertain to references in Part II, entitled "The Biochemistry of Photoreception."

[a] Apparently the only other isomer capable of combining with the protein (in the absence, of course, of any isomerase) is the 6-mono-*cis* The conjugate is called "isorhodopsin "

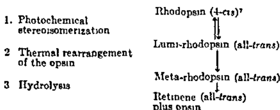
Goldsmith (*65*) found retinene$_1$ in the heads of honeybees A photosensitive pigment in the compound eye of the drone has a maximum at the unusually low wavelength of 440 mμ Two sensitivity maxima were found in the ocelli of the worker, at 490, and between 335 and 340 mμ. Since retinene itself has a maximum above 370 mμ, one may speculate whether an unusual *cis*-form may not be present

In a survey of the visual pigments Crescitelli (*66*) concludes that while there is simplicity and generality in terms of carotenoid (i e, retinene$_1$ or retinene$_2$), versatility and biological usefulness is expressed in the protein moieties which emerge as the purveyors of biological specificity The interesting observation of Dowling and Wald (*67*) should be added, that opsin disappears in severe nutritional night blindness. The attachment of the retinene "has a powerful stabilizing effect on this protein"

Kropf and Hubbard (*68*) suggest that the red color of rhodopsin is due to the interaction of the carbonyl of retinene with a specific amino group of opsin to form a Schiff base, and that negatively charged groups located elsewhere on the protein "stabilize the resonance forms of the chromophore that constitute its first excited state" Because this lowers the energy of transition between ground and first excited state, they reason that the yellow color is shifted to red, i e, from that of retinene to rhodopsin

It is difficult in a brief commentary to do justice to Rushton's paper (*69*) on the kinetics of cone pigments The normal fovea contains two pigments, one of which is the protanope pigment (presumed to be the pigment upon which the brightness or lightness factor is assessed, maximum at 540 mμ) and the other is more red sensitive (maximum at 590 mμ)

We shall consider, first, vision at low light intensities. The rhodopsin, or visual purple, is bleached by light and passes through orange-red intermediates (lumi- and meta-rhodopsins) to form the yellow all-*trans* retinene and rod opsin (scotopsin) This was summarized as follows by Hubbard *et al* (*70*):



[7] The Geneva numbering is used here

The all-*trans* retinene then may either be isomerized to the 4-mono-*cis* (neo-b) aldehyde by retinene isomerase or converted to the all-*trans* alcohol by retinene reductase This is then isomerized and converted to neo-b-retinene by an alcohol dehydrogenase, and when recombined with scotopsin, it regenerates visual purple. The retinene reductase operates in conjunction with reduced DPN.

Essentially the same general system may be assumed to operate in cone vision and for the chicken pigment, iodopsin, the corresponding lumi- and meta-intermediates were found (*70*).

It is however more complicated since the cones provide our perception of color Individuals with normal color vision are said to be trichromats Judd points out (*71*) that to give a complete color response, at least three cones must feed impulses along three independent nerve fibers, and at some stage, either in the retina or in the brain these impulses must be combined From the fact that three independent items are derived from the cones (light-dark, red-green, yellow-blue) there must be at least three cone photopigments, or photopigment filter combinations. Finally, Judd remarks that *we know closely how their ability to absorb* radiant energy must vary with wavelength Maxima are shown around 450, 550, and 600 mμ Relative spectral sensitivity curves given by Judd for rods and cones show maxima at 510 and 555 mμ respectively. The value 510 mμ is, however, too high according to Wald and Brown (*72*). The difference spectra of human rhodopsin in aqueous solution and in rod particles, suspended at random in the presence of hydroxylamine, *show λ maxima at 493 and 500 mμ respectively* The absorption spectrum of the rods is then compared with the scotopic luminosity function, quantized, and corrected for ocular transmission which then represents the sensitivity at the retinal surface of a lensless eye, and the two are then shown to correspond exactly Wald and Brown point out that "this correspondence hangs upon a peculiar phenomenon the displacement of the absorption spectrum of rhodopsin (from 493 mμ) toward the red in the outer segments of the rods, as compared with the spectrum in aqueous solution In the highly organized microstructure of the outer segments, the molecules of rhodopsin are fixed in position and highly oriented"

The change from cone to rod vision, from that of the day to the night necessitates a shift to a photoreceptor less sensitive to the red Consequently red appears black at night, whereas blue becomes gray (the Purkinje effect).

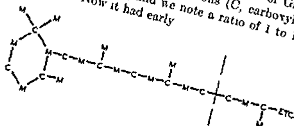
The provision for three photoreceptor pigments presents no insuperable barrier One might have different protein conjugates with the same retinene isomer, or the same protein with different retinene isomers.

Proof, however, might be very difficult to obtain in the latter case Regardless of the condition in which the retinene-protein conjugate might exist *in situ*, it might be extractable only in one form as a result of rapid conversion to the most stable conjugate

VII. Biogenesis of Carotenoids

A Origin of the Carbons

On the basis of acetate-carbon incorporation into carotene in a mold *Mucor hiemalis*, we may commence with the work of Grob and Butler (*73*) and identify the origin of the carbons (C, carboxyl, M, methyl), as shown in Formula (VIII), and we note a ratio of 1 to 1 5 in favor of incorporation of M Now it had early



(VIII)

been shown that another mold *Phycomyces blakesleeanus* (*74*) in culture media preferentially drew on nonsugar carbon for its carotene, even though the bulk of the carbon came from the sugar In media labeled with glucose in which 90% of the carbon was glucose-carbon, the specific activity of the carotene carbon was invariably lower than that of the medium-carbon

We need therefore to survey a wider field, the origin of isoprenoid-carbon in general, to determine whether there is in fact one C_5 repeating unit for the wide variety of compounds which we consider related to isoprene

In the last ten years, a characteristic C_5 repeating unit of the typical isoprenoid compound has variously been sought in β-methylcrotonate (*75*), leucine (*76*), hydroxymethylglutarate (HMG) (*77*), mevalonic acid (*78–80*) and, to complete the circle, methylcrotonate again (*81*)

Unquestionably mevalonic acid holds the center of the stage at this time The biggest difficulties lie in the absence of proved intermediates between it and the C_{40} carotenoid molecule, and in lack of proof that the unit is incorporated intact

Had leucine-4-C^{14} been tested before leucine-2-C^{14}, the high degree of incorporation of the former would conform with an anticipated result

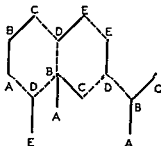
and the conclusion might well have been reached that after decarboxylation, the remaining five carbons of leucine were incorporated as a unit.

In the case of irregularly linked C_5 units in terpenes, listed by Haagen-Smit (*82*), it is indeed simpler to postulate that the end product is the result of numbers of C_2 and C_3 fragments combining as in macrolide structures (*83*)



(IX)

where, for example C_3 fragments may combine head to center, and one has only to assume some linkages of this type to make sense of such "irregular" structures as elemol, eremophilone, or abietic acid A tortuous isoprene pattern is thus eliminated for abietic acid, or a virtually impossible one for eremophilone,



(X)

Here, we have joined one of the carbons of each C_2 fragment to the *center* of one, or to the *end* of another C_3 fragment, or to another C_2, which is routine for some macrolides. Admittedly, this sort of exercise has no value except when a labeled substrate is to be checked for the location of some specific atom The fact is, however, that an extraordinary diversity of compounds must be accounted for, and the list is steadily growing, the latest important additions being ubiquinone (*84*) and a quinone from alfalfa with coenzyme-Q-like activity (*85*) Consequently, flexibility is *attained if we recognize the ability of the body* to cope with C_3 and C_2 fragments in more than one way.

Reports from various laboratories steadily strengthen the over-all scheme shown in Fig 1 The C-4 of leucine occupies position 3 in HMG, and it does not matter how this recycles with the acetoacetate pool, this carbon has least chance of being diluted out Designating x for the

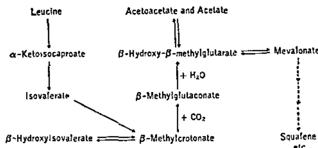
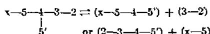


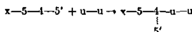
FIG 1 Leucine and the acetoacetate pool interrelationships

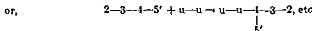
CO_2 fixed with the β-methylcrotonate, u for carbon of unknown origin in the acetate pool, and using the leucine numbering, we may construct our HMG from the various carbons in this manner

1. HMG (leucine numbering) recycles with the acetoacetate pool, via the CoA derivatives



2 The two types of acetoacetate revert to HMG, as for example





If (x—5—4—5′) breaks up into two acetates, there is the chance of the 4-carbon moving to a terminal position, e g, u—u—5′—4; however the reaction acetoacetate → acetate would appear to be slow, compared with the recycling of HMG with acetoacetate and acetate, and thus we can explain rather more than twice the degree of incorporation into carotene of the 4-carbon of leucine compared with the 3-carbon

Bloch (*86*) in 1944 had postulated that isovalerate was an intermediate in leucine metabolism Coon and Gurin (*87*) showed that the α and β carbons of leucine gave a 2-carbon fragment capable of condensing to acetoacetate Zabin and Bloch (*88*) obtained C_2 and C_3 fragments from isovalerate both of which could be metabolized to acetoacetate Their C_3 fragment yielded ketone bodies more readily and less CO_2 than the C_2 fragment This is in line with our own results, the CO_2 yield falling off rapidly from C-1 to C-4 in leucine (*89*, *90*).

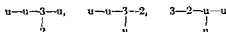
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The scheme postulated gives us a number of possible starting points for the C_5 isoprenoid unit. Leucine, whether 2-, 3-, or 4-labeled, gives rise to a prominent labeled zone for HMG; the C-3 is incorporated with around 50% the effectiveness of the C-4. Hence if hydroxyisovalerate or methylcrotonate is to be considered the precursor, it can only be as a result of a back reaction from HMG with recycling to cause dilution of carbons 2 and 3 of leucine

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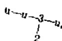
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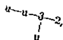
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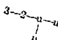
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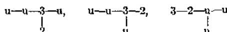
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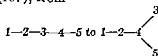
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by Mase (*81*) indicate no preferential effect of mevalonic acid over β-methylcrotonate in carotene synthesis in *Penicillium sclerotiorum.* If one is prepared to consider all these C_5 and C_6 compounds as potential contributors to a common take-off point, with C_3 and C_2 fragments recycling, to some extent independently, it becomes possible to reconcile in substantial measure the conflicting data

Location of the label when acetate or mevalonate is injected into ripening tomato fruit shows very high activity in a compound with a maximum at 231 mμ (*105*). Recent evidence (*106*) favors continued consideration of this compound as an intermediate in carotenoid synthesis. The difference in ability of various organisms to handle mevalonate and methylcrotonate is to be noted in this abstract, also.

The ease or difficulty with which HMG, for example, may contribute directly C_3 and C_2 fragments to, or indirectly promote the synthesis of, some C_5 intermediate such as α-hydroxy-β-methyl acetoacetate—CoA, or tiglyl-CoA, in any given organism would determine the observed response. Thus HMG does promote carotene biosynthesis in *Phycomyces in vivo,* whereas mevalonate does not

When one considers the extraordinary rearrangement of glutamic acid carbons by *Clostridia* (*107*), from



it seems probable that *in vitro* results may simulate but not duplicate the behavior of the living organism in its handling of these fragments Furthermore, one maintains much more flexibility with respect to the whole gamut of isoprenoid compounds which plants and microorganisms are capable of synthesizing

Nevertheless at this time, it would seem probable that mevalonate is the immediate precursor of the C_5 repeating unit for many isoprenoid structures, that it is most adequately established for squalene, and somewhat less unambiguously for several of the others

VIII. The Biochemical Sequence in C_{40} Conversions

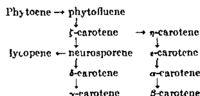
A. General

The first reasonably comprehensive sequence to be postulated is known by the names of its proponents, Porter and Lincoln (*108*). Based in large measure upon spectroscopic evidence and genetic studies on tomatoes, it assumes that carotene formation proceeds by a series of regular stepwise dehydrogenations from a compound with a maximum

around 230 mμ (tetrahydrophytoene, the formula of which was set at $C_{40}H_{70}$) through phytoene, maximum 285 mμ, phytofluene, ζ-carotene, neurosporene, and lycopene ($C_{40}H_{56}$). The foregoing are all aliphatic, and by ring closure the alicyclic carotenoids would then arise This required modification when it was found by Rabourn and Quackenbush (*4*) that phytoene had the formula $C_{40}H_{64}$, and was in fact an *octa*hydrolycopene with nine double bonds, the central three of which were in conjugation.

The formulation of sequences is based on various considerations, including findings of heritable differences in tomato fruits (*109–112*), changes in composition of the pigments in various molds, *Phycomyces* (*113, 114*), *Penicillium sclerotiorum* (*115*), *Neurospora* (*116, 117*), in *Corynebacteria* (*118*), in *Chlorella* mutants (*119–121*) and the detailed kinetic study by Jensen *et al* (*122*) of the effect of diphenylamine on pigmentation in *Rhodospirillum*. The foregoing are among the more important references in this field and they must be scrutinized to determine what features they offer in common and what individual features may be irrelevant with respect to any common pattern of carotenoid development

Rabourn (*109*) postulated a thoroughgoing revision of the Porter-Lincoln sequence which has been presented in detail by Mase (*81*) The revised scheme has three branch points

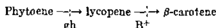


One branch point is at neurosporene, from which either lycopene or δ- and γ-carotenes are derived δ-Carotene differs from γ-carotene not by two hydrogens, but by an α-configuration in the ring Then at ζ-carotene a major choice is possible, leading either to aliphatic or alicyclic polyenes. In this case, α-carotene is presumed to precede β-carotene, which is contradictory to the Cholnoky view (*37*), and probably also to that of Booth (*123*) Leaves of white-rooted fodder carrots contain no α-carotene, but are normal in total carotene content This is also true of so-called albinos from colored varieties (the leaves are a normal green and contain no α-carotene), and of the wild carrot

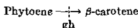
Neurosporene is represented in this scheme as a dihydrolycopene, whereas the preponderance of evidence (*124*) makes $C_{40}H_{60}$ more probable This is an objection as to detail, not necessarily to the principle.

B. Inheritance of Carotenoid Differences

Dealing first with the tomato, we may show that except for one mutant, ghost, an albino (*111*), the pigment complex of the photosynthetic tissues of numerous mutants shows no qualitative differences of any significance regardless of whether the fruit ultimately synthesizes very little or a great deal of carotenoid (5 versus 150 to 200 μg per gram). The following genes, when homozygous, cause *formation in the ripe* tomato of predominantly these pigments: ghost, phytoene; tangerine, prolycopene and ζ-carotene; beta-orange, β-carotene; normal fruit, all-*trans* lycopene; strains relatively high in δ-carotene have also been obtained at Purdue However, while such data provide valuable clues, their interpretation is open to question Fruit of ghost, red, and beta-orange types differ in the condition of two genes, B and gh^{+}. Thus we might well postulate the biochemical sequence.



However, if gh is introduced into beta-orange, the evidence disappears as we find



In this connection Zalokar (*116*) raises the question whether in differently pigmented mutants, the genes act directly or indirectly. He argues that the existence of an albino mutant in which phytoene is found does not necessarily require a direct block in the conversion of phytoene to colored polyenes The same effect might be obtained by a deficiency in the oxidation mechanism, preventing conversion of unknown and more saturated precursors into the less saturated colored carotenoids He cites the effects of lack of oxygen, and of diphenylamine in *Neurospora* The disappearance of phytoene could not be related to the formation of any particular carotenoid He had earlier (*117*) made a detailed study of rates of formation of phytoene, an acidic pigment, the members of the Porter and Lincoln series, and γ-carotene and spirilloxanthin, in *Neurospora crassa* after exposure to light and oxygen Measurements were made over a 24-hour period, at hourly intervals for the first three hours ζ-Carotene, neurosporene, and lycopene all appeared simultaneously and at the same rate. Except for phytoene and the acidic pigment, Zalokar concluded that there was independent formation of the different pigments.

C The Diphenylamine Effect

The introduction of diphenylamine (DPA), into cultures of *Mycobacterium* and other organisms causes a shift in the type of polyene produced Turian (*125*) and Turian and Haxo (*126*) working with *Mycobacterium phlei* and in *Neurospora* revealed the presence of more saturated polyenes, and deduced that these may represent intermediates which would normally remain undetected

Strong evidence for this reasoning has been provided by Jensen *et al.* (*122*) who used *Rhodospirillum rubrum* in which the carotenoid synthesis characteristic of the organism had been disrupted by addition of DPA The DPA was then washed out, the centrifuged and thoroughly washed cells being resuspended in buffer and incubated anaerobically in the light The data show unequivocally a stoichiometric relationship between disappearance of phytofluene, ζ-carotene, etc , and formation of the more highly colored compounds The monohydroxy derivatives of the various hydrocarbons, with the exception of phytoene, were detected. Of the suggestions, the most plausible is that these derivatives are side-products in the main biosynthetic sequence

Rhodospirillum produces no alicyclic carotenoids, so far as can be ascertained, and the abnormal production of polyene in the form of phytoene whenever normal synthesis of other polyenes is hampered leaves two open questions—whether phytoene is a precursor of phytofluene, and what are the precursors of the alicyclic series

D Chlorella Mutants

Claes (*119–121*) described four *Chlorella* mutants obtained by X-ray treatment which she contrasted with the wild type The last mentioned contained the usual mixture of chlorophylls *a* and *b*, α- and β-carotenes, and the oxygenated xanthophylls In the mutants, there appeared several more saturated hydrocarbons not found in the wild type, namely, phytoene, phytofluene, and ζ-carotene and the normal pigments One strain contained only phytoene in reduced amount, or not at all In one instance, there was a complete block of xanthophyll, in another xanthophylls were formed, but not α- or β-carotenes These strains could only be grown in the dark, with added glucose The fourth strain, however, could be grown in the light or dark, and the pigment complex depended on the choice In the dark, the more saturated types were found, as well as lycopene, whereas in the light, the mutant behaved like the wild type In a second paper (*120*) the presence of lycopene in both the all-*trans* and a poly-*cis* form, prolycopene, is confirmed, as well as a poly-*cis* neurosporene. In the dark, the *cis*-forms appear. With blue light, these are converted to

the respective all-*trans* isomers, while in blue and red light, the pigmentation is that of the normal wild type.

Claes and Nakayama (*127*) have recently shown for one mutant that the *cis*-forms are converted to the all-*trans* isomers in red light provided the irradiation is performed in a nitrogen atmosphere. They suggest that the stereoconfiguration of the carotenoids in the chloroplast might be controlled by the chlorophyll.

However, in dealing with the anomaly presented by the strain which synthesizes wild-type pigments in red light, Claes points out that the plastid structures of the mutants are no longer normal Consequently changes on illumination after culturing in the dark do not prove that events are sequential and causally related

E. Effects of Thiamine and Temperature

The level of thiamine (*118*) in the culture medium of *Corynebacterium* sp determines whether in addition to the aliphatic spirilloxanthin and lycoxanthin (typical of red cells, with low thiamine) the alicyclic cryptoxanthin will be formed (orange-yellow cells, with high thiamine). A similar color change is observable in *Rhodotorula penaeus* (*128*) associated with temperature At 5°, the predominant pigment is β-carotene; at 20–25°, it is torularhodin

F General Considerations

Two major questions to be considered are the relationship of phytoene to the phytofluene-lycopene series, and whether in fact the alicyclic compounds stem from ζ-carotene The latter question is confused by uncertainty as to identity

pounds in the ζ-fract

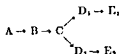
Haxo (*130*) who des

Neurospora The other, similar to flavacin in absorption spectrum, appears to be a pigment X of Claes (*121*), possibly the η-carotene of Rabourn In our own experiments with *Syncephalastrum*, this seems to be the pigment detected when β-carotene synthesis is partially restricted (*131*), e g., by the use of methylheptenone, when the β-carotene falls from 3300 μg per gram to about 2000, and the phytoene rises from 50 to 100 up to 1000 μg per gram, and the Porter-Lincoln series of aliphatic compounds does not appear. Equally difficult to reconcile with the Rabourn revision of the Porter-Lincoln sequence is the effect of starvation, followed by incubation in the presence of glucose, as an energy source (*132*). Starvation uniformly caused a drop in β-carotene content, also in phytoene and phytofluene Treated with diphenylamine in the energy-rich

transfer medium, there was a net gain in phytoene, a net loss in phytofluene, and restoration of β-carotene to nearly the prestarvation level.

The problem in elucidating the sequences involved is well illustrated in two recent papers Lynen *et al* (*133*) have shown that the phosphorylated derivative of mevalonate yields, in the presence of Mg^{++}, ATP, and yeast enzymes, 3-methyl Δ^3-butenyl-1-pyrophosphate, "active isoprene." This condenses to geranyl pyrophosphate (C_{10}) from which monoterpenes may be derived, or to farnesyl pyrophosphate (C_{15}) for sesquiterpenes. Addition of one more C_5 unit would yield a geranylgeraniol (C_{20}). The tail-to-tail linkage of two C_{15} or two C_{20} units would provide the structures for squalene or carotenoid as the case might be Purcell *et al* (*134*) have reported additional data on the incorporation of mevalonate into tomato carotenoids They deduce from the activities that neither phytoene nor lycopene can be precursors of β-carotene, the carefully purified phytoene being "essentially free of radioactivity" A definitive appraisal must await further details In order of increasing adsorbability we have the sequence β- and ζ-carotenes, and lycopene Tables I and III of their report show a rapid falling off in the total activity assigned to these fractions This general distribution of activity on the column parallels that found in the writer's laboratory, with *Phycomyces* extracts, high in the β- and ζ-region, with a rapid falling off as elution is continued

Let us consider two possibilities (1) that A is transformed to E, via intermediates B, C and D, (2) that a branchpoint exists at C, and that E_1 is formed via D_1, and E_2 via D_2, i e

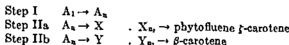


Where A is labeled, in a single-dose experiment, the postulated sequences cannot be established from the activities unless we know the turnover. We cannot even predict *a priori* that E_1 and E_2 both deriving from C will have the same specific activities, though we may anticipate that the ratio of the total counts in D_1 and E_1, versus the total counts for D_2 and E_2 would approximate the corresponding ratio for the quantities formed during the experiment

The dilemma then is that if we are to accept our hypothetical substrate A as the precursor for E_1 and E_2, both of which are found in considerable quantity, it is difficult to explain why they should differ in specific activity by more than two orders of magnitude In this instance E_1 and E_2 may be taken to represent β-carotene and lycopene respectively, and A, mevalonate There must therefore be some revision either

of experimentally found differences in activities or of concepts as to how aliphatic and alicyclic carotenoids arise

We see therefore that to make any broad generalizations, the findings on many different organisms must be assessed. We once postulated a very general scheme as follows (*135*):



This formalized scheme states merely that step I is common to all subsequent carotenoid syntheses At A_n, there is a branch, one path leads to aliphatic, the other to alicyclic forms. As a result of the work from Stanier's laboratory, step IIa can clearly be extended to lycopene and spirilloxanthin.

Results with cell-free preparations have so far had modest success Until the details for improved yields have been worked out, starvation of mold cultures, followed by transfer to an energy-rich medium and a labeled substrate under test would seem one promising line of attack. The use of double labeled substrates would also yield more precise information as to the point A_n where the pathways diverge, if indeed they do, a view held by the writer.

Addendum

A substantial number of phosphorylated intermediates between mevalonate and squalene have now been isolated and characterized (*136–141*) as a result of intensive investigation It was pointed out in Bloch's laboratory (*136, 137*) that the "biological isoprene unit" which undergoes condensation, possessed terminal methylene groups, and that it arose from mevalonate via the phosphorylated derivative

Lynen and co-workers (*138, 139*) postulate the following scheme from mevalonate: from MVA to the 5-phosphate, then to the 5-pyrophosphate, and thence to isopentenylpyrophosphate (IPP), all three steps being mediated by ATP. IPP, in the presence of its isomerase, is converted to γ, γ-dimethylallylpyrophosphate which can condense with more IPP to give first the geranyl derivative, then the farnesyl. The last-mentioned requires TPNH for conversion to squalene Witting and Porter (*140, 141*) have similarly found a number of intermediates, and have identified geraniol as a component. They suggest that Lynen's scheme may be oversimplified as Ogilvie (*142*) has characterized one intermediate as 4-carboxyfarnesol. This raises some doubt as to whether the dimethylallylpyrophosphate is on the direct pathway.

An immediate parallel between sterol and carotenoid syntheses cannot yet be demonstrated, as phytol $C_{20}H_{39}OH$ is not homologous with geraniol $C_{10}H_{17}OH$ and farnesol $C_{15}H_{25}OH$. This does not preclude discovery of the homologous $C_{20}H_{33}OH$ by means of gas chromatography and other powerful tools now available However, the C_{40} carotenoid might arise by addition of terminal C_5 units to squalene (acted upon by a squalene isomerase) or to a C_{30} squalene precursor When the mechanism for the formation of the C_{40} molecule has been established, it will be relatively simple to explain the effects of genetic factors which control carotenoid pigmentation

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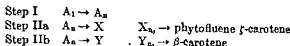
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